

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM
HUNGARICAE

ADIUVANTIBUS

I. DÖMÖK, E. FARKAS, J. HORVÁTH, S. KOTLÁN,
R. MANNINGER, I. NÁSZ, A. PELC, K. RAUSS, J. SZIRMAI,
J. WEISSFEILER

REDIGIT

G. IVÁNOVICS

TOMUS XIV

FASCICULUS I



AKADÉMIAI KIADÓ, BUDAPEST

1967

ACTA MICROBIOL. ACAD. SCI. HUNG.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA
MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: BUDAPEST V., ALKOTMÁNY UTCA 21.

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Acta Microbiologica, Budapest, Keleti Postahivatal, Postafiók 64.

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The rate of subscription to the *Acta Microbiologica* is 165 forints a volume. Orders may be placed with “Kultúra” Foreign Trade Company for Books and Newspapers (Budapest I., Fő utca 32. Account No. 43-790-057-181) or with representatives abroad.

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FORMAMIDE BREAKDOWN REACTION FOR THE DIFFERENTIATION OF ENTEROBACTERIACEAE

By

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(Received May 11, 1966)

Summary. The simple alkalization test allows to divide the *Enterobacteriaceae* into quick formamide decomposing organisms (all *Morganella* and part of *Klebsiella* and *Retzgerella* strains), slow formamide decomposing organisms (*Serratia* strains) and formamide negative bacteria (other *Enterobacteriaceae*). None of the examined *Enterobacteriaceae* cultures attacked the following amino group-containing substances: dimethylformamide, para-aminosalicylic acid, para-aminobenzoic acid, beta-naphthyl-amine, ortho-phenylenediamine, aniline, diethylamine, urethane, hexamethylenetetramine.

Bacterial enzymes liberate ammonia from certain amino compounds and the resulting change in the pH of the medium can be detected by the alkalization test [6].

Materials and methods

The alkalization test was carried out in the following medium. Distilled water, 100 ml; substrate, 0.20 g; KH_2PO_4 , 0.10 g; K_2HPO_4 , 0.05 g; freshly prepared phenol red solution (phenolsulphonphthalein, 0.01 g; N NaOH, 0.40 ml; distilled water, 4.6 ml), 2.5 ml; pH 5.6. Sterilization at 115 °C for 15 minutes. On the day of the examination the media were distributed in Jena or Unihost test tubes (16 × 160 mm) in 2 ml portions for the macrotest and in 0.5 ml portions for the microtest. (The tubes cleaned previously from traces of acid or alkali, were stoppered with artificial cotton and sterilized.) A two-loopful amount of a one-day-old culture was suspended in the medium. The used loop was 2 mm in diameter. The inoculated macrotest media were incubated at 37 °C for 7 days. Readings were made daily. Tubes with the microtest media were incubated in a water bath at 40 to 41 °C for 4 hours, then in a 37 °C thermostat overnight. Readings were made after 4 hours and on the next day.

Results are shown in the Tables. For a more detailed technique of the test see references 6 and 7.

Slight changes in the colour of the indicator (weakly positive and negative reactions) could be distinguished more easily if before inoculation the medium had been supplemented with 0.01 g per 100 ml of fluorescein.

Results

Alkalization in formamide medium. Of the examined strains those belonging to *Morganella* attacked formamide most readily. All strains of this group showed rapid, strongly positive reactions (Table I). Part of *Klebsiella* and *Retzgerella* strains also reacted positively. Some *Serratia* cultures gave a late positive reaction. With the microtest a smaller number of positive reactions were obtained and alkalization was less intensive.

Table I

*Enteric bacteria causing alkaline reaction
in formamide medium*
Macrotest. Amount of bacteria, two loopful

	No. of strains	(+)	+	Among positives	
				(++)	++
<i>Klebsiella</i>	68	6	13	3	13
<i>Serratia</i>	42	17	—	—	—
<i>Morganella</i>	43	—	43	—	43
<i>Rettgerella</i>	29	—	9	5	2

Key: (+) = Red colouration within 4–7 days
 + = Red colouration within 3 days
 (++) = Intensive red colouration within 4–7 days
 ++ = Intensive red colouration within 3 days

None of the examined 125 *E. coli*, 143 *Salmonella*, 25 *Enterobacter*, 18 *Citrobacter*, 17 *Arizona*, 212 *Shigella*, 30 *P. vulgaris*, 30 *P. mirabilis*, 30 *Providencia* and 4 *Hafnia* strains showed alkaline reaction in formamide medium.

The highly active cultures seemed suitable for demonstrating the effect of inhibitors. One hour before inoculation, the medium containing 4×10^{-2} μ M/ml formamide was provided with 10^{-1} μ M/ml streptomycin or 2×10^{-4} μ M/ml ethylmercurisalicylate. As shown in Table II the inhibitory effect varied with different cultures. Ethylmercurisalicylate was a more effective inhibitor. Similar results were observed with the microtest.

Table II

*Effect of inhibitors on alkalization by Morganella
and Klebsiella in formamide medium*
Macrotest. Amount of bacteria, two loopful

	Inhibitor	—	(+)	+	Among positives	
					(++)	++
<i>Morganella</i>	—	—	—	24	—	24
	Streptomycin	9	—	15	3	2
	Ethylmercurisalicylate	21	1	2	—	—
<i>Klebsiella</i>	—	—	—	13	3	10
	Streptomycin	2	3	8	4	1
	Ethylmercurisalicylate	13	—	—	—	—

Key: — = No red colouration within 7 days
 (+) = Red colouration within 4–7 days
 + = Red colouration within 3 days
 (++) = Intensive red colouration within 4–7 days
 ++ = Intensive red colouration within 3 days

All *Morganella* cultures used in experiments with inhibitors were resistant to streptomycin. However, according to the degree of streptomycin resistance the strains fell into two groups. Six highly resistant strains which multiplied in the presence of $2 \times 10^{-1} \mu\text{M/ml}$ streptomycin, gave a positive formamide reaction in a medium containing $2 \times 10^{-1} \mu\text{M/ml}$ antibiotic. Eight out of 18 less resistant strains which were inhibited by $3.2 \times 10^{-4} \mu\text{M/ml}$ streptomycin, gave a negative formamide reaction in the presence of $10^{-1} \mu\text{M/ml}$ of streptomycin. Thus a certain parallelism seems to exist between streptomycin resistance and inhibition of the amidase of *Morganella* strains. The thermoresistance of *Morganella* amidase was examined by performing the microtest in a water bath at temperatures of 39–41, 45–47 and 51–53°C. A variable but generally moderate inhibition was observed with 12 cultures at 51–53°C.

The results have shown that the formamide reaction is suitable for the identification of *Morganella*, which always gives a rapid, strongly positive alkaline reaction. Similar results were observed with certain *Klebsiella* and *Retterella* strains. Accordingly, these organisms can be divided into biotypes by the formamide test. It is interesting that bacteria of the *Serratia* group, which readily attack glycine [6] and different peptones and display an intensive endogenous oxidation [7], were generally inert in formamide breakdown. In contrast, *Morganella* strains showed the very opposite of this behaviour. This character is suitable for the identification of bacteria belonging to these two groups.

Alkalization in media containing other amido compounds. The 816 enteric bacteria examined in the present study were tested for activity against dimethylformamide, para-aminosalicylic acid, para-aminobenzoic acid, beta-naphthylamine, ortho-phenylenediamine, aniline, diethylamine, urethane, and hexamethylenetetramine. None of these substances were attacked by the examined organisms, or at most a weak alkalization attributable to endogeneous respiration was observed.

Discussion

In higher animal organisms two enzymes are responsible for the oxidation of amine compounds. The oxidative deamination of monoamides is catalyzed by amine oxidase, that of diamines by diamine oxidase. The substrate accepts oxygen and releases ammonia and is therefore transformed into the corresponding aldehyde [2]. While the amino oxidases of mammals have been widely investigated, little is known about the breakdown of amines by bacteria. WERLE [8] showed that by microorganisms (*E. coli*, *Ps. aeruginosa*, *Ps. fluorescens*) cadaverine was more rapidly decomposed than histamine. GALE [2] demonstrated that the washed suspension of *Ps. aeruginosa* produced ammonia by the oxidative breakdown of putrescine, cadaverine, agmatine, histamine and tyramine. Some *E. coli* strains oxidized tyramine and/or putrescine and cadaverine. The enzymic activity, which was never strong, had a pH optimum

of 7.5 to 9.5. The oxygen consumption of freshly isolated strains of the above organisms was increased by ethylamine, iso-amylamine, phenylethylamine and aniline; this finding is probably not attributable to the oxidation of the amines but to a catalytic effect on endogeneous respiration. ZELLER *et al.* [1, 9] have concluded that bacteria contain only one well-defined enzyme acting upon amines. This enzyme, diamine oxidase, is especially active in various *Pseudomonas* species and *M. smegmatis*. Diamine oxidases present in bacteria and in mammals are not identical. Mammal diamine oxidases are inhibited by certain guanidine compounds, while the corresponding bacterial enzyme by streptomycin. *M. smegmatis* is able to bring about an oxidative deamination of ethylenediamine, 1,3-trimethylenediamine, 1,3-diaminopropanol, 1,3-diaminobutane, putrescine, cadaverine and hexamethylenediamine. Putrescine, 1,3-diaminopropanol and trimethyldiamine are most readily attacked by this bacterium. Streptomycin inhibits the enzyme responsible for this reaction by a direct effect [9]. In the present study some parallelism was observed between enzyme inhibition and antibacterial activity. Suspensions of *Achromobacter* strains oxidize putrescine, histamine and iso-amylamine; the bacterial diamine oxidase may deaminate also monoamines [1]. Recently an amidase has been isolated from *Ps. aeruginosa* which showed 5 different types of activity [4].

As far as it is known to the author, the present work is the first to describe the breakdown of amino and amido compounds as a diagnostic test. Since these substances are easy to obtain at low prices, the test can be introduced in routine laboratories without difficulty. The intensive breakdown of formamide by all *Morganella* and some *Rettingerella* and *Klebsiella* strains is noticeable, as the reaction proceeds at pH 5.6, which is considerably more acid than the optimal reaction.

With this simple procedure, large numbers of strains can be tested and strains with different activity can be selected for studies on bacterial enzymes.

The considerable difference between the activity against formamide and dimethylformamide is an interesting example of enzymic specificity. Many similar observations have been made *viz.* D-amino acid deaminases are unable to deaminate N-dimethylamino acids [3], or animal glycine deaminase does not attack N-dimethylglycine [5].

Analogous observations make it probable that the alkaline reaction produced as a result of the catalysing activity of bacterial deaminases is due to ammonia released in the reaction.

Acknowledgement. The author is indebted for *Enterobacteriaceae* strains to Drs. V. KERESZ (Institute of Microbiology, Hygienic Faculty, Charles University, Prague), E. ALDOVÁ (Institute of Epidemiology and Microbiology, Prague), F. KOLTA (Chief, Public Health Laboratory, Tatabánya), and further to Dr. G. HABÁN, Chief, Department of Bacteriology, Dr. H. MILCH, Head, Phage Laboratory, Dr. B. LÁNYI, Head, Culture Collection, and Drs. Z. DEÁK and V. LÁSZLÓ, Phage Laboratory, National Institute of Public Health, Budapest.

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ANTIBIOTIC PRODUCTION OF HYPHAL FRACTIONS OF STREPTOMYCES GRISEUS

I. DETERMINATION OF STREPTOMYCIN PRODUCING ACTIVITY OF WASHED MYCELIUM IN SHORT-TERM INCUBATION

By

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(Received July 23, 1966)

Summary. Streptomycin producing activity of washed mycelium of *Streptomyces griseus* strain 52-1 shaken for 2 to 3 hours in glucose-free soybean-cornsteep medium has been determined.

Under the experimental conditions used glucose inhibited the production of streptomycin.

The amount of streptomycin adsorbed on the mycelium during fermentation was determined after washing in phosphate buffer. Washed mycelium exerted 30 to 50 per cent of streptomycin producing activity found for mycelium subcultured in the original medium.

Disintegration in Waring blender increased the rate of streptomycin production.

Mechanism of, and influence of incubation and life cycle on, antibiotic production are important theoretical and industrial problems. They have been approached e. g. by measuring the streptomycin producing activity (SPA) of washed mycelium in defined medium [3-6]. It has been demonstrated this way that certain substances act as precursors, inhibitors or stimulators (glucose, inositol, arginine, streptidine, etc.).

The purpose of the present investigation was to elaborate a method which supplies information concerning the actual SPA in short-term incubation. Since our further aim is an estimation of the SPA of different hyphal fractions, the effect of disintegration in Waring blender has also been examined.

Materials and methods

Culture method. Streptomycin-producing *Streptomyces griseus* strain 52-1 was used. Maintenance of the strain and shaken cultures was made in soybean-cornsteep liquor medium [7]. The experiments were performed with mycelium grown in 45 hour shaken cultures. Each experiment was repeated three times; as most results were closely similar, the result of one will only be presented.

The mycelium was washed three times in *M*/15 pH 7.6 phosphate buffer at 0 °C. The cultures were incubated at 2 to 3 ml final volumes in 17 ml ampoules (5.2 × 2.4 cm) at 27 °C. Shaking was performed as described in reference 7. Unless otherwise indicated, the medium was glucose-free soybean-cornsteep medium at a twofold final dilution. The system contained 0.5 to 3 mg per ml of dry weight mycelium.

Streptomycin assay was performed by the agar diffusion technique with strain *B. subtilis* ATCC 6633. As glucose at a concentration of more than 0.05 per cent decreased the sensitivity to 84 per cent (unpublished observation), data obtained in media containing glucose

were corrected. The samples were diluted with *M*/15 pH 7.6 buffer and 0.1 ml aliquots were pipetted into the holes. On a 18 × 24 cm agar plate 41 holes were prepared. Each standard dilution (1, 2 and 4 µg/ml) was measured in 7, each sample in 4 holes.

Disintegration in Waring blender. The mycelium was suspended in distilled water at 10 mg dry weight per ml; of this 12 ml aliquots were placed in 25 ml MSE "special universal container" and disintegrated at 14000 r. p. m.

Determination of dry weight was performed by pipetting 0.5 to 1 ml suspension on membrane filter, washing with approximately 15 ml twice distilled water, and drying at 70 °C overnight. The difference in the weight of the membrane filter before and after filtration corresponded to the dry weight of the mycelium.

Extraction with oxalic acid. After washing in phosphate buffer, a dense aqueous suspension of the mycelium was adjusted with 5 per cent oxalic acid to pH 2.5 and incubated for 1 ½ hours at 60 °C. The mycelium was then centrifuged, the supernatant was adjusted with NaOH to pH 7.6 and used for streptomycin assay.

Results

In the first step of the experiments it has been found that SPA highly depends on the medium employed. After washing in phosphate buffer the mycelium was resuspended so as to correspond in dry weight to the mycelium content of the original fermentation liquor. Streptomycin production was also calculated for ml of the original fermentation medium (Table I).

SPA determination was performed in twice diluted glucose-free soybean medium which had been found most suitable in the above experiments. It is seen that streptomycin production was inhibited by glucose proportionally to its concentration.

Assay of the streptomycin production rate is reliable only if the washed mycelium contains no antibiotic or its streptomycin content is negligible as compared to the amount of streptomycin produced during 2 hours of incubation.

Table I

*Streptomycin producing activity of Streptomyces griseus strain 52-1 mycelium in different media grown in 45-hour submerged culture**

Incubation medium	Streptomycin production, µg/ml	
	2 hours	8 hours
2 × diluted soy medium + 1% glucose	26.6	55.2
2 × diluted soy medium + 0.25% glucose	24.3	62.0
2 × diluted soy medium without glucose	45.0	81.0
<i>M</i> /60 phosphate buffer + 1% glucose	—	12.5
<i>M</i> /60 phosphate buffer + 0.5% glucose	—	13.3

* Streptomycin content of fermentation liquor, 83 µg/ml.

In order to determine the amount of streptomycin remaining in the mycelium after washing 3 times in phosphate buffer, a dense suspension (7 to

9 mg per ml of dry weight mycelium) was extracted with oxalic acid and parallelly after 2 1/2 hours incubation streptomycin production was determined.

At the beginning of shaking the mycelium contained only 12.4 per cent of the streptomycin content found after 2 1/2 hours incubation (Table II).

Table II

*Streptomycin content of Streptomyces griseus strain 52-1 mycelium after washing three times in phosphate buffer**

System	Streptomycin, μg/mg mycelium
Supernatant after oxalic acid extraction	0.5
Streptomycin produced after shaking for 2 1/2 hours	4.0
Oxalic acid adjusted with NaOH to pH 7.6 . .	0.0
Streptomycin (3.13 μg/ml) after treatment with oxalic acid	3.2**

* Streptomycin content of fermentation liquor, 163 μg/ml

** μg/ml.

In subsequent experiments the SPA of the original culture and of the washed suspension incubated in the medium mentioned, was compared. A 45-hour culture was divided into two parts. One part was incubated further under the same conditions, the other was washed in the usual manner in phosphate buffer. The system contained the same amount of mycelium as the original culture. Results are presented in Table III.

Table III

Effect of washing in phosphate buffer on streptomycin producing activity of Streptomyces griseus strain 52-1 mycelium after cultivation for 45 hours

Time of sampling	Streptomycin, μg/ml		
	Original culture		Washed mycelium
	Total content	Difference to 0-time content*	
0	187	0	0
2 hr.	207	20	11.8
4 hr. 40 min.	.	.	21.5
5 hr. 40 min.	243	56	.
8 hr.	262	75	29.6

* Amount of streptomycin produced in addition to the streptomycin content of the 45-hour culture.

Fig. 1 indicates the rate of streptomycin formation expressed as the amount of antibiotic produced per hour. The values were calculated by dividing the amount of streptomycin produced between each sampling with the corresponding time.

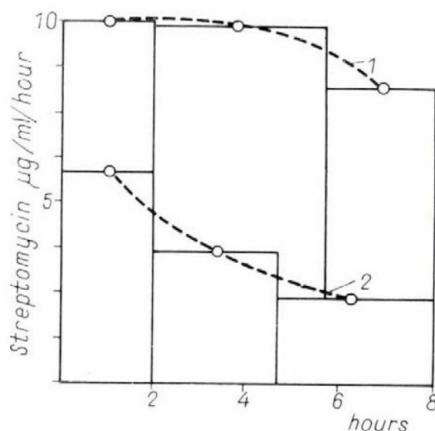


Fig. 1. Rate of streptomycin formation by *Streptomyces griseus* strain 52-1 mycelium washed in phosphate buffer. 1: Production rate in original culture between 45 and 54 hours; 2: Production rate in shaken twice-diluted glucose-free soy medium after washing with phosphate buffer

Under the experimental conditions used, streptomycin produced by washed mycelium amounted to 30 to 50 per cent of the rate obtained in the original fermentation. During the 8-hour incubation period the rate of strepto-

Table IV

Effect of disintegration on the streptomycin producing activity of *Streptomyces griseus* strain 52-1 mycelium

System	Streptomycin production after 2 hr. 20 min.; µg/mg mycelium
Intact mycelium, suspended in distilled water	4.83
Intact mycelium suspended in supernatant of blended culture	4.47
Disintegrated mycelium suspended in supernatant of blended culture.....	6.82
Disintegrated mycelium washed 3 times and resuspended in distilled water.....	6.50
Supernatant of blended culture	0.0
Supernatant of blended culture*	0.16

* Direct assay of concentrated supernatant; the result is expressed in dry weight of the corresponding disintegrated suspension.

mycin production decreased in both cultures. As the observed differences were small, no final conclusions can be drawn as to the original fermentation. However, in view of the 72-hour production of our culture (approximately 350 $\mu\text{g/ml}$), the data seem reliable.

Finally, the effect of disintegration in the Waring blender on SPA of mycelium was examined. A 45-hour culture was washed in phosphate buffer, resuspended in distilled water and divided into two parts. One part was treated in the Waring blender, the other served as control. If needed, centrifugation of the aqueous suspension was repeated and the sediment was resuspended in the appropriate medium. Results are shown in Table IV.

The SPA of mycelium increased after blending. This finding could not be attributed to the effect of some non-sedimenting substance produced on disintegration (lysis) of the mycelium. The SPA of disintegrated mycelium did not decrease after removal of the supernatant; on the other hand, the activity of intact mycelium did not increase after addition of the supernatant.

Discussion

According to the findings, it is possible to determine the SPA of mycelium in short incubation periods. In the experiments glucose was found to inhibit the formation of streptomycin (Table I). This finding is in agreement with that of MAJUMDAR [3], who observed that in the presence of glucose no streptomycin was produced in 9 hours. The stimulatory effect of glucose in yeast extract medium used by NÖMI [4] is not incongruent with our findings, since it appeared only after 48 or 72 hours of shaking and the 24-hour values were identical or, more frequently, lower than those obtained in the glucose-free control medium.

According to HOCKENHULL [1] phosphates above a certain concentration (0.2 to 0.4 g/l) decrease streptomycin production. Despite of this observation, we used phosphate buffer for the washing of mycelium, because in this manner streptomycin was effectively extracted [4]. In our incubation system the phosphate concentration was below the inhibitory level described by HOCKENHULL. It may thus be supposed that under our experimental conditions washing in phosphate buffer did not affect streptomycin production, and only a negligible amount of streptomycin remained in the mycelium after washing (Table II). The increase of the streptomycin level on short-term incubation indicated that the formation of streptomycin molecule from biologically inactive or less active precursors is a rapid process, which can be followed in short intervals, although washing decreases the rate of production. In our opinion short-term incubation determinations characterize SPA better than estimations performed after shaking for long periods, which may cause

important biological and life cycle changes. This consideration is supported by the opposite effect of glucose in the two kinds of technique.

Disintegration in the Waring blender, which breaks the filaments into hyphal fractions 10 to 50 μ in length [8], increased SPA (Table IV). Blending caused about 5 per cent lysis, but the experiments have shown that the increase in SPA was not due to the effect of some activating agent liberated during disintegration. The cause of this is not clear. A closer contact between biologically different hyphal pieces and consequently a more favourable condition for mutual effects between filaments may have been responsible for the higher SPA values. A stopping or decrease of supply transport, which has been shown to take place along the hyphae of fungi [2], may also be associated with the finding. A detailed examination of the phenomenon will be the subject of a subsequent paper.

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IMMUNOFLUORESCENT STUDIES ON YEASTS

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(Received August 22, 1966)

Summary. Immunofluorescent staining with anti-albicans conjugate of 107 strains belonging to 102 different yeast species and indirect staining of 13 strains of 7 yeast species have been carried out. It has been shown that by properly diluted conjugates and immune sera *Procandida albicans* and *Procandida stellatoidea* can be differentiated from other yeasts. A comparison of immunofluorescent results with the accepted diagnostic criteria indicated that the immunofluorescent method may be suitable for elucidating taxonomic problems.

Introduction into medical mycology of the immunofluorescent technique elaborated by COONS *et al.* [1—3] has recently been attempted by some investigators [4—9]. The close antigenic relationships between different yeast species [10—17] caused, however, many cross reactions and thus limited the application of the method [18—20]. In spite of these difficulties it seemed desirable to make further investigations, because immunofluorescent tracing has the advantage of simplicity and rapidity over the classical methods [21—23]. In the present experiments we have attempted to gain information as to the influence of antigenic relationships on the result of immunofluorescent examinations and as to improvements needed to make the present methods suitable for the rapid diagnosis of yeasts. *Procandida albicans* (syn. *Candida albicans*) [26] the commonest pathogenic yeast species in this country has been used as the model organism.

Materials and methods

Direct staining. Anti-albicans immune serum was prepared in rabbits with *Pc. albicans* strain 85/1957 isolated from clinical material in our laboratory. Rabbits tested previously with tube agglutination for the absence of *Pc. albicans* antibodies, were immunized intravenously with formalized suspensions containing 5×10^8 cells per ml. The animals received at two-day intervals 0.5 ml doses in the first, 1.0 ml doses in the second, and 1.5 ml doses in the third, fourth and fifth week of immunization. In the sixth week the rabbits were bled by cardiac puncture. The centrifuged serum was inactivated in the usual manner and preserved with 0.5 per cent phenol. Agglutinin titre determination was carried out by tube agglutination with a formalized suspension of the homologous strain. The 1 : 640 titre whole serum was conjugated with Rhodamine B 200 fluorescent dye as described by CHADWICK [27].

The above conjugate was used for the examination of 107 strains of 102 different yeast species. The organisms were cultured on CSILLAG's molasses agar [28] for 48 hours. Smears were fixed with methanol for 2 minutes, then treated for 20 minutes at room temperature with undiluted and 1 : 10, 1 : 20, 1 : 40 and 1 : 80 diluted conjugate. Finally the smears were washed in running tap water for 2 to 3 minutes. Readings were performed by means of a Leitz Ortho-

lux II microscope under blue light at $250\times$ magnification. Intensity of fluorescence was compared to the fluorescence of *Pc. albicans* cells treated with the undiluted conjugate (++++).

Indirect staining. Sera were prepared from *Pc. albicans* strains 178/1960 and 360/1960, isolated from clinical material in our laboratory. Immunization was performed as described for direct staining, with the exception that the rabbits were bled two weeks after the last injection. Thus sera exhibiting 1 : 5120 and 1 : 2560 tube agglutination titres were obtained. The smears were fixed as described above, then treated with appropriate dilutions of the sera at room temperature for 20 minutes. After washing in running tap water for 2 to 3 minutes the smears were treated with fluorescein-isothiocyanate-conjugated anti-rabbit goat immune globulin for 20 minutes at room temperature and washed again in running tap water for 2 to 3 minutes. Evaluation of the results was performed as described for direct staining.

Results

Table I summarizes the results of direct staining. In order to make the results clearer, the examined organisms are listed within every group of reaction according to the order described in our classification [23, 26].

From Table I it is seen that the examined yeasts gave different reactions with the labelled anti-albicans serum. Some of them stained weakly or not at all even by the undiluted conjugate; others exhibited a strong fluorescence

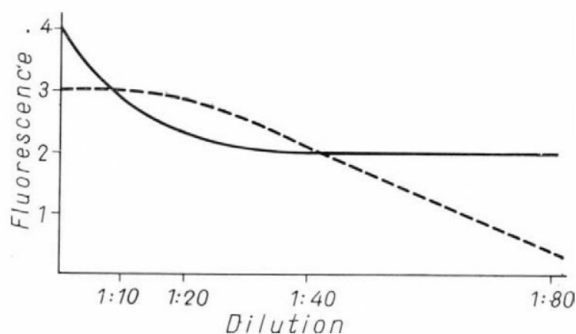


Fig. 1. Relationship between the fluorescence of yeasts and the dilution of anti-albicans conjugate used for staining. — Lower limit of fluorescence of *Pc. albicans* strains. — — — Upper limit of fluorescence of other yeasts (except *Pc. stellatoidea*). Figures on the ordinate indicate the degree of fluorescence

with 1 : 40 diluted serum almost similar to, or even identical with, the fluorescence of the homologous strain (++ reaction). When serum dilutions of 1 : 80 were used, in addition to *Procandida albicans* only *Pc. stellatoidea* showed a well-defined reaction, that is, by diluting the conjugate 1 : 80, the fluorescence of every yeast but *Pc. albicans* and *Pc. stellatoidea* could be eliminated. The fluorescence of the homologous organism (*Pc. albicans*) also decreased with dilution of the serum, but to a lesser degree than that of other strains. Thus at dilutions of 1 : 80 the difference between the fluorescence of *Pc. albicans* and other strains was more marked than that observed with the undiluted conjugate. Fig. 1 shows the fluorescence at different serum dilutions of *Pc. albicans* and *Pc. stellatoidea* as compared to that of other species.

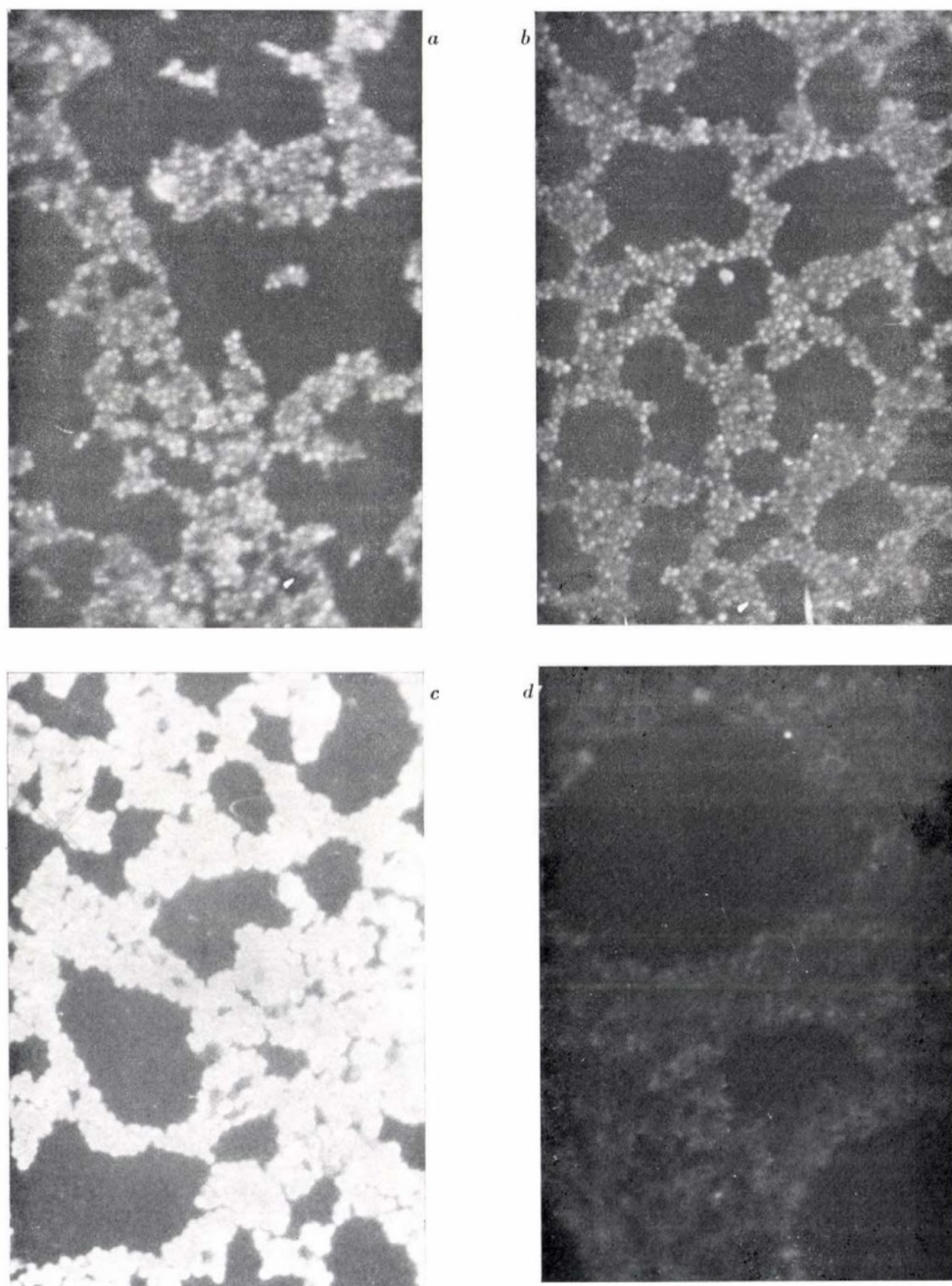


Fig. 2. Fluorescence of different yeasts stained with anti-albicans conjugate 85/1957
(a) *Procandida albicans* strain 85/1957 stained with anti-albicans conjugate diluted 1 : 80;
(b) *Pc. albicans* strain 187/1960 stained with anti-albicans conjugate diluted 1 : 80; (c) *Pc. albicans* strain 85/1957 stained with undiluted anti-albicans conjugate; (d) *Azymocandida ingens* stained with anti-albicans conjugate diluted 1 : 80

Table I

Immunofluorescent reaction with anti-albicans serum of different yeast species
(Direct staining, serum 85/1957)

Degree of fluorescence with dilution of conjugate					Species
Conc.	1 : 10	1 : 20	1 : 40	1 : 80	
— (±)	— —	— —	— —	— (—)	<i>Endomycopsis wickerhamii</i> , <i>Prosporbolomyces hispanicus</i> , <i>Psp. salmonicolor</i> , <i>Geotrichum rotundum</i> , <i>G. amycelium</i> , <i>Nigrococcus nigricans</i>
		negative			
+	—	—	—	—	<i>Nadsonia slovakia</i> , <i>Schizosaccharomyces octosporus</i> , <i>Saccharomyces terricolus</i> , <i>S. oviformis</i> , <i>Endomycopsis subpelliculosa</i> , <i>Trichosporon cutaneum</i> , <i>Tr. undulatum</i> , <i>Procandida grubyi</i> , <i>Brettanomyces custersii</i> , <i>Torulopsis inconspicua</i> , <i>Paratorulopsis aerea</i>
(+)	±	—	—	(—)	
		very weak			
++	+	—	—	—	<i>Endomycopsis reessii</i> , <i>Azymomyces vanriji</i> , <i>Saccharomyces carlsbergensis</i> , <i>Debaryomyces vini</i> , <i>Vanderwaltia vineae</i> , <i>Zymopichia quercibus</i> , <i>Zp. saitoi</i> , <i>Hansenula beijerinckii</i> , <i>H. schneeggii</i> , <i>Candida benhamii</i> , <i>Candida</i> sp. 355/60, <i>Torulopsis vanzyllii</i> , <i>T. westerdijkii</i> , <i>Rhodotorula flava</i>
(++)	+	±	—	(—)	
(+)	+	±	—	(—)	
		weak			
+++	++	+	—	—	<i>Saccharomyces acidifaciens</i> , <i>S. pastorianus</i> , <i>S. robertsii</i> , <i>S. pretoriensis</i> , <i>Dekkermomyces delphensis</i> , <i>Geotrichum linkii</i> , <i>G. matalense</i> , <i>Azymoprocandida japonica</i> , <i>Azymocandida zeylanoides</i> , <i>Ac. ingens</i> , <i>Ac. corniculata</i> , <i>Ac. muscorum</i> (2), <i>Candida slooffii</i> , <i>C. solani</i> , <i>C. utilis</i> , <i>C. guilliermondii</i> , <i>Candida</i> sp. VIII/61, <i>Torulopsis cantarelli</i> , <i>T. gropengiesseri</i> , <i>T. magnoliae</i> , <i>T. capsuligenus</i> , <i>Torulopsis</i> sp. 263/62, <i>Paratorulopsis pinus</i> , <i>Pt. banhegyii</i> , <i>Cryptococcus neoformans</i> , <i>Cr. diffluens</i> , <i>Cr. laurentii</i> , <i>Cr. albidus</i> , <i>Rhodotorula slooffii</i> , <i>Rh. minuta</i> , <i>Rh. mucilaginosus</i> , <i>Rhodotorula</i> sp. 235/57, <i>Rhodotorula</i> sp. P.S.
(+++)	++	+	±	(—)	
(++)	++	+	—	(—)	
(++)	+	+	±	(—)	
		medium			
+++	+++	++	+	—	<i>Saccharomyces transvaalensis</i> , <i>S. bayanus</i> , <i>S. cerevisiae</i> , <i>S. cerevisiae</i> var. <i>ellips.</i> , <i>Saccharomyces</i> sp. 559/60, <i>Debaryomyces kloederi</i> , <i>Zymodebaryomyces globosus</i> , <i>Hansenula anomala</i> , <i>Kluyveromyces polysporus</i> , <i>Kl. africanus</i> , <i>Dekkermomyces lodderi</i> , <i>D. marxianus</i> , <i>D. fragilis</i> (2), <i>Geotrichum suaveolens</i> , <i>Procandida tropicalis</i> , <i>Azymocandida rugosa</i> , <i>Ac. aaseri</i> , <i>Ac. curvata</i> , <i>Ac. mycoderma</i> , <i>Candida krusei</i> , <i>C. norvegensis</i> , <i>C. requinnyi</i> , <i>C. soosii</i> , <i>C. brum-</i>
(+++)	+++	++	+	(±)	
(+++)	++	++	+	(±)	
		good			

Table I continued

Degree of fluorescence with dilution of conjugate					Species
Conc.	1 : 10	1 : 20	1 : 40	1 : 80	
		good			<i>ptii</i> , <i>C. reukaufii</i> , <i>C. pulcherrima</i> , <i>C. parapsilosis</i> , <i>C. clausenii</i> , <i>C. flareri</i> , <i>C. beverwijkii</i> , <i>C. pseudotropicalis</i> , <i>Paratorulopsis domerqii</i> , <i>Rhodotorula pallida</i> , <i>Rh. glutinis</i>
++++ (+++)	+++ +++	++ ++	++ ++	+ (+)	<i>Geotrichum candidum</i> , <i>G. hirtum</i>
		strong			
++++ (+++)	++++ +++	+++ +++	++ ++	++ (++)	<i>Procandida stellatoidea</i> , <i>Pc. albicans</i> (4)
(++++)	+++	++	++	++	
		very strong			

Nomenclature and order of species is given according to the system of NOVÁK and ZSOLT [23, 26]. Bracketed figures mean the number of strains if more than one was examined.

Fig. 2 demonstrates fluorescent micrographs of the *Pc. albicans* strain used for immunization, a further *Pc. albicans* strain and *Azymocandida ingens*. The smears were stained with anti-albicans conjugate diluted 1 : 80. A smear stained with undiluted conjugate is also shown. It should be noted that *Pc. albicans* and *Pc. stellatoidea* exhibited an orange coloured fluorescence even at dilutions 1 : 80, and could therefore be well distinguished from other yeasts the $\pm$ degree weak fluorescence of which at this dilution was rather a greyish-green autofluorescent reaction.

Table I indicates a considerable difference between various species belonging to the same genus.

Results obtained with indirect staining are presented in Tables II and III. It is evident that the association of the degree of fluorescence with the serum dilution is proportional with the titre of the serum and, on the other hand, the specificity improves when the indirect method is used. In our examinations only *Pc. stellatoidea* gave similar or identical results with *Pc. albicans*.

Discussion

The results of the present investigations should be considered with respect to the rapid practical diagnosis of *Pc. albicans* and the yeast taxonomic use of the immunofluorescent technique.

As to practical purposes, it has been shown that the specificity of both direct and indirect staining can be increased by diluting the conjugate and the

Table II

Fluorescence of smears stained by the indirect method
(Titre of anti-albicans serum 1 : 2560)

Species	Strain	Dilution of anti-albicans serum 360/1960				
		1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
<i>Procandida albicans</i>	360/1960	++++	++++	++++	++++	+++
	431/1960	++++	++++	++++	+++	+
	66/1960	++++	++++	++++	++++	++
	178/1960	++++	++++	++++	+++	+
	85/1957	++++	++++	++++	++++	++
<i>Geotrichum candidum</i>	1	++	+	+	+	±
<i>Trichosporon cutaneum</i>	199/1958	++	+	—	—	—

Table III

Fluorescence of smears stained by the indirect method
(Titre of anti-albicans serum, 1 : 5120)

Species	Strain	Dilution of anti-albicans serum 178/1960		
		1 : 40	1 : 80	1 : 160
<i>Procandida albicans</i>	178/1960	++++	++++	++++
	66/1960	++++	++++	+++
	360/1960	++++	+++	+++
	431/1960	++++	+++	+++
	29/1966	++++	++++	++++
	126/1966	++++	++++	+++
<i>Procandida stellatoidea</i>	29—64—4	+++	+++	+++
<i>Procandida tropicalis</i>	337/1964	++	++	±
<i>Candida claussenii</i>	460-sect/1961	+++	++	+
<i>Rhodotorula glutinis</i>	518p/1959	+	+	±

serum. Thus the complicated procedure of antibody absorption can be omitted. Dilution results in the elimination of cross reactions with other yeasts except *Pc. stellatoidea* and therefore the method seems adequate for the rapid diagnosis of *Pc. albicans*. With respect to *Pc. stellatoidea*, which is systematically distinct from *Pc. albicans*, it should be noted that because of their almost identical antigenic structure, it is difficult to eliminate the cross reaction between these

Table IV
Comparison of antigenic structure and fluorescence of yeasts

Species	Antigenic structure*	Degree of fluorescence with dilution of conjugate				
		Conc.	1 : 10	1 : 20	1 : 40	1 : 80
<i>S. oviformis</i>	1, 2, 3, 10, 14, 18, 31	+	—	—	—	—
<i>S. cerevisiae</i>	1, 2, 3, (10), (14), 18, (31), a, e	+++	+++	++	+	±
<i>S. cerevisiae</i> var. <i>ellips.</i>	1, 2, 3, (10), (14), 18, (31), a, e	+++	+++	++	+	±
<i>S. carlsbergensis</i>	1, 8, 10, 28, 32	++	±	—	—	—
<i>E. subpelliculosa</i>	1, 2, 14, 15, (16), 20	+	±	±	—	—
<i>H. anomala</i>	1, 2, 14, 15, 16, 20	+++	++	++	+	±
<i>D. marxianus</i>	1, 8, (10), a	++	++	++	+	±
<i>D. fragilis</i>	1, 8, (10), 28, 31, a	+++	+++	++	+	±
<i>D. fragilis</i>	1, 8, (10), 28, 31, a	+++	+++	++	++	±
<i>Pc. stellatoidea</i>	1, 2, 3, 4, 5, (10)	+++	+++	+++	++	++
<i>Pc. albicans</i> **	1, 2, 3, 4, 5, 6, 7	++++	++++	+++	++	++
<i>Pc. albicans</i>	1, 2, 3, 4, 5, 6, 7	++++	+++	++	++	++
<i>Pc. albicans</i>	1, 2, 3, 4, 5, 6, 7	++++	++++	+++	++	++
<i>Pc. tropicalis</i>	1, 2, 3, 4, 5, 6	+++	++	++	+	±
<i>A. zeylanoides</i>	1, 2, 3, 4, 13, 17, (a), c, h	++	++	+	+	±
<i>A. mycoderma</i>	1, 2, 5, 11, 12	+++	+++	+++	+	±
<i>A. rugosa</i>	1, 2, 3, 4, 11, 19, g	+++	++	++	+	±
<i>C. krusei</i>	1, 2, 5, (11), b	+++	++	++	+	±
<i>C. reukaufii</i>	1, 2, (5), (11), (12), b, f	+++	++	++	+	±
<i>C. pulcherrima</i>	1, 2, 3, 5, 13, 14, (15), d	+++	+++	++	+	±
<i>C. parapsilosis</i>	1, 2, 3, 5, (13), (14), (15), c	+++	++	++	+	±
<i>C. claussenii</i>	1, 2, 3, 4, 5, 6, 7	++	++	++	+	±
<i>C. utilis</i>	(1), (2), (14), 16, 17, c	++	+	+	±	±
<i>C. guilliermondii</i>	1, 2, 3, 4, 9	++	+	+	+	±
<i>C. pseudotropicalis</i>	1, 8, (10), 28, 31, a	+++	++	++	+	±
<i>T. inconspicua</i>	1, 2, 5, 11, 12	+	—	—	—	—

* Figures stand for thermostable, letters for thermolabile partial antigens.

** Strain used for the production of immune serum.

organisms. The low incidence of *Pc. stellatoidea* [21, 25] lessens the danger of misidentification, which may also occur when the usual identification method based on the examination of chlamydospore production is used without testing sucrose assimilation [24].

With the immunofluorescent procedure, the dilution of unabsorbed conjugates or sera provides a quantitative method similar to tube agglutination. The degree of fluorescence is probably influenced not only by the qualitative composition of the antigen mosaic, but also by the quantitative distribution of partial antigens. In order to demonstrate this, the known antigenic structure of some species [10–17] and the degree of fluorescence are compared in Table IV. It is seen that *Candida guilliermondii* shares 4, while *C. krusei* only 3 partial antigens with *Pc. albicans*; yet, *C. krusei* reacted more strongly with the anti-*albicans* conjugate. A similar finding was revealed for *Saccharomyces oviformis* and *S. cerevisiae* as, although both organisms contained 3 antigens present also in *Pc. albicans*, *S. cerevisiae* reacted more definitely.

Small variations in the fluorescence of *Pc. albicans* strains may be due to differences in the strains, in storage in the laboratory, and, although positive and negative controls were always used, to subjective errors in reading.

Conjugate dilution 1:80 and serum dilutions 1:80 and 1:160 found suitable for direct and indirect staining, respectively, apply only to our sera showing 1:640, 1:2560 and 1:5120 titres. Since higher titre sera may occur (we were able to produce a serum with a titre higher than 1:5120), the working dilution of the serum should always be determined empirically. The degree of dilution is, of course, influenced by an absorption with *Pc. stellatoidea* prior to labelling or indirect staining, since absorption decreases the cross reaction also with other species.

As in these studies usually one strain was only examined from each species, from the taxonomic point of view these studies should be regarded as orientation experiments. Some data, however, deserve mentioning.

It was interesting that some organisms (*Prosporobolomyces hispanicus*, *Psp. salmonicolor*, *Geotrichum rotundatum*, *G. amycelium*, *Nigroccoccus nigricans*) did not react even with the undiluted conjugate. This finding supports the view that the morphological relationship of Sporomycetaceae and *N. nigricans* with other yeasts is only partial. The taxonomic relationship between *Geotrichum* species described by CARETTA [29–32] (*G. hirtum*, *G. suaveolens*, *G. rotundum* and *G. amycelium*) and other species that have been included among yeasts on the basis of arthrospore production (*G. linkii*, *G. candidum* and *G. matalense*) is not yet elucidated. Our experiments indicate that the immunofluorescent technique may be useful for throwing light on such problems, since two out of CARETTA's species failed to react with anti-*albicans* conjugate, although at least one antigen (thermostable factor No. 1) seems to be present in all yeasts.

In comparison with the accepted taxonomic criteria, our results seem to confirm the view that fermentation reactions are not quite suitable for the classification of yeasts [23] and indicate that differentiation based on sugar and nitrate assimilation and pseudomycelium production is more valuable. This consideration has been supported by the following findings. *Saccharomyces*

terricola differs in fluorescence more definitely from *S. acidifaciens* and *S. transvaalensis* than the two latter from each other. *S. acidifaciens* and *S. transvaalensis* differ only in the fermentation of galactose while *S. terricola* differs from both organisms in that it does not assimilate galactose. Among yeasts belonging to the *S. pastorianus* group [23], pseudomycelium-non-producing *S. oviformis* differs in fluorescence more definitely from pseudomycelium-producing *S. bayanus* and *S. pastorianus* than the two latter organisms from each other. *Pichia saitoi* described by KODAMA *et al.* [33] is hardly distinguishable from *Zymopichia quercibus* on the basis of taxonomic properties; this is supported by immunofluorescent results. In view of the fermentation pattern of *P. saitoi*, this organism can be included in the genus *Zymopichia* of our system [26]. On the basis of its other properties, this yeast belongs to the species group *Z. pastori* [23]. Thus it is proposed that without altering the original description, this organism should be reclassified under the name *Zymopichia saitoi* *comb. nov.* Of two other *Hansenula* species differing in raffinose assimilation and raffinose fermentation from pseudomycelium-non-producing *Hansenula schneegii*, the likewise pseudomycelium negative *H. beijerinckii* exhibited a fluorescence similar to that of *H. schneegii*. Of the three *Geotrichum* species (*G. linkii*, *G. candidum*, *G. matalense*) included in our system [26], *G. linkii* and *G. matalense* gave a similar fluorescent reaction; these two organisms had been shown to be more closely related to each other than to *G. candidum* [34]. In the *Procandida* genus the chlamydospore-producing *Pc. stellatoidea* and *Pc. albicans* gave identical fluorescence, but considerably differed from the other two *Procandida* species. *Azymocandida aaseri*, *Ac. curvata*, *Ac. corniculata* and *Ac. muscorum* displayed an identical behaviour not only in nitrate assimilation but also in fluorescence. The results of organisms belonging to species groups *Candida krusei* (*C. slooffii*, *C. krusei*, *C. norvegensis*) and *Candida requinyii* (*C. requinyii*, *C. soosii*) [23] were very similar within the species group. It should also be mentioned that two *Candida* species (*C. reukaufii* and *C. pulcherrima*) recently transferred by WICKERHAM [35] into the genus *Chlamydozoma* were very similar in fluorescence although they are considerably different in some properties (production of pulcherrin pigment and propeller-shaped grouping of cells).

With the above examples we have attempted to show that the immunofluorescent technique may be useful for taxonomic differentiation provided that more extensive studies are carried out on an adequate number of strains. The method might then call attention to the necessity of uniting certain species [23].

LITERATURE

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CHROMATOGRAPHIC CHARACTERIZATION OF ECHOVIRUS PROTOTYPE STRAINS

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(Received August 29, 1966)

Summary. Elution from DEAE columns of prototype strains of echoviruses has been studied. Elution was performed with NaCl solutions of increasing molarity. Strains were characterized on the basis of the NaCl molarity resulting in the elution of the bulk of the virus. By this method the strains could be divided into 3 groups, according to their exhibiting elution peaks at low (< 0.01), intermediate ($0.01-0.09$), and high (> 0.09) NaCl molarities. Group 1 comprised prototype strains 1, 8 and 12; group 2 strains 1 (2nd peak), 4, 7, 13, 14, 20, 22, 24 and 29; and group 3 strains 2, 3, 5, 6, 9, 11, 15, 16, 17, 18, 19, 21, 23, 25, 26, 27, 30 and 32. The importance of chromatographic analysis in the detection of intratypic characters is discussed.

In a previous paper [1] we reported on the chromatographic analysis of type 3 poliovirus strains isolated from humans prior and after vaccination with type 3 live poliovirus vaccine. The method proved to be suitable for the characterization of intratypic features in that it permitted differentiation of wild and attenuated strains. This observation suggested the possibility of intratypic differentiation of further enteroviruses.

The present paper reports on the chromatography of prototype strains of echoviruses.

Materials and methods

The strains used are listed in Table I.

Identity of the strains was checked by neutralization test performed prior to chromatography. Type sera were supplied by the Institute of Hygiene, University of Freiburg, Germany, and by the Department of Virology, National Institute of Public Health, Budapest.

Cultivation and preparation of strains for chromatography. Primary monkey kidney cells were grown in Kolle flasks of 70 sq. mc. surface for 7 days, to obtain confluent cell sheets. The growth medium consisted of HANKS' balanced salt solution supplemented with 0.25 per cent lactalbumin hydrolysate and 5 per cent calf serum. The 7-day old cultures were inoculated with 1 ml of undiluted virus suspension. The infected cultures were incubated in PARKER'S No. 199 medium containing 0.25 per cent lactalbumin hydrolysate, at 37°C . Cultures exhibiting complete cytopathic changes were frozen at -20°C . After thawing, the material was centrifuged at 15,000 r. p. m. for 30 minutes at $+5^{\circ}\text{C}$. The supernatants were harvested and dialysed against 0.02 M phosphate buffer (pH 7.5) at 5°C .

Chromatography on DEAE cellulose column. The principles of THOMSEN'S method [2] were followed. DEAE cellulose (MN 2100, capacity about 0.7 meq/g) was decanted twice with 0.02 M pH 7.5 phosphate buffer. The columns prepared were 6 cm high and 1 cm in diameter. Adsorption was done at room temperature, using 2 ml of stock virus suspension (fraction E_0). The individual eluents were applied in 2 ml volumes and collected in two fractions of 1 ml. The first eluent was 0.02 M pH 7.5 phosphate buffer (fraction E_1 , E_2). The further eluents con-

Table I
List of prototype strains studied

Echovirus type	Designation of strain	Passage number	Source
1	Farouk	4	Budapest
2	Cornelis	3	Freiburg
3	Morrissey	3	Budapest
4	Pesascek	7	Budapest
5	Noyce	3	Budapest
6	D'Amori	5	Freiburg
7	Wallace	3	Freiburg
8	Bryson	4	Freiburg
9	Hill	5	Freiburg
9	TN 200	7	Freiburg
11	Gregory	4	Freiburg
12	Travis	4	Freiburg
13	Del Carmen	2	Budapest
14	Tow	10	Budapest
15	CH 96-51	6	Budapest
16	Harrington	3	Budapest
17	CHHE-29	6	Budapest
18	Metcalf	1	Budapest
19	Burke	—	Freiburg
20	JV-1	5	Freiburg
21	Farina	5	Freiburg
22	Harris	10	Freiburg
23	Williamson	8	Budapest
24	De Camp	5	Budapest
25	JV-4	5	Budapest
26	Coronel	4	Budapest
27	Bacon	8	Budapest
29	JV-10	3	Budapest
30	Bastianni	3	Budapest
32	PR-10	3	Budapest

tained also NaCl in increasing molar concentrations according to the gradient, 0.025, 0.05, 0.075, 0.10, 0.15, 0.20, 0.30, 0.40, 0.50, and 0.60 (fractions E_{3-22}). The NaCl contents of the eluate fractions were, in fraction $E_6 < 0.01 M$; in fractions E_7 to E_{22} 0.01, 0.015, 0.026, 0.04, 0.05, 0.06, 0.074, 0.09, 0.12, 0.16, 0.17, 0.20, 0.26, 0.30, 0.36 and 0.42 M , respectively, as shown in a preliminary model experiment. These values were then considered reference standards.

The fractions were stored at -20°C until virus assay.

Virus assay. Virus titrations were made in primary rhesus monkey kidney tube cultures by the serial dilution method. Titres were read after 3 and 7 days of incubation. Estimation of the titre was based on the 7th day reading. Titre values were computed by the method of REED and MUENCH and expressed in terms of $\log \text{CPD}_{50}$.

Results

Results of chromatographic analysis of the echovirus prototype strains listed in Table I, are presented in Table II.

The figures shown in Table II represent the virus titres of the individual fractions. Peak values are framed.

All elution peaks were below 0.3 M NaCl. No regularity was detectable in the localization of the peaks of the individual prototype strains. This observation suggested the existence of a certain difference as compared to the adsorption of polioviruses to $\text{Al}(\text{OH})_3$ gel [3]. Therefore, the classification of echovirus prototype strains according to their adsorption to DEAE cellulose was carried out on an arbitrary basis. Strains 1, 8 and 12 had elution maxima at NaCl concentrations lower than 0.01 M. These were considered group 1. Group 2 comprised the strains with elution maxima from 0.01 to 0.09 M NaCl, *viz.* strains 1 (second peak), 4, 7, 13, 14, 20, 22, 24 and 29. With group 3 were classified all strains with elution maxima above 0.09 M NaCl, *viz.* 2, 3, 5, 6, 9 (Hill), 9 (TM 200), 11, 15, 16, 17, 18, 19, 21, 23, 25, 26, 27, 30 and 32. It should be noted that prototype strain 1 had two approximately identical elution peaks in the 3rd and 10th fractions. Also, two peaks were exhibited by strains 3, 7 and 12. These peaks, however, differed by several orders of magnitude. The majority of peaks for all the strains tested, was at the higher molar concentrations of NaCl, 50 per cent of the total belonging to this group, whereas the other 50 per cent comprised both the low and intermediate molarities. On comparison of the stock virus titre to the amount of virus recovered in the eluates, 80–100 per cent average yields were observed. Thus, the experimental conditions used ensured a practically complete recovery of adsorbed virions.

Discussion

Echovirus strains of one and the same serological type may considerably differ in antigenic characters. Strains exhibiting relatively high antigenic capacities were designated as "prime" strains [4]. Of these, the strains belonging to type 6 have already been studied in detail [5]. Strains isolated from aseptic meningitis patients were poorly neutralized by homologous as well as prototype sera (S phase). After a few passages in monkey kidney tissue culture, however, very satisfactory neutralization titres were obtained (phase B).

Intratypic differences were detected not only in the neutralization test, but also in the haemagglutinating activity of the appropriate strains (6, 7, 8, 9, 10). SIMON and DÖMÖK [11, 12] separated by chromatography on Ca-phosphate column haemagglutinating (H^+) and non-haemagglutinating (H^-) virions from a population of type 6 echoviruses isolated from humans. The pure lines selected by the above method differed in their further characters, but

Table II
Elution of echovirus prototype strains

Echovirus		Virus content of the elution											
type	strain	0*	1	2	3	4	5	6 0.01**	7 0.01	8 0.015	9 0.026	10 0.04	11 0.05
1	Farouk	3.25	6.0	5.75	6.25	5.75	5.75	5.5	5.5	4.75	5.75	6.5	5.5
2	Corneils	0	0	0	2.25	3.0	3.0	3.75	3.5	3.75	3.0	3.5	4.0
3	Morrissey	0	0	0	2.5	0	0	0	0	0	0	2.75	3.5
4	Pesascek	0	0	0	0	2.5	2.75	3.0	3.0	3.25	4.25	4.75	5.75
5	Noyce	0	0	0	0	0	0	0	0	0	0	0	0
6	D'Amori	0	0	0	0	0	0	0	0	0	2.0	2.5	3.0
7	Wallace	0	0	0	2.5	2.5	4.5	4.5	5.5	5.5	7.0	4.25	5.75
8	Bryson	0	2.75	5.75	6.75	6.75	5.25	4.25	4.25	4.25	4.5	4.5	3.75
9	Hill	0	0	0	2.0	2.5	4.0	3.75	4.0	4.75	5.25	4.75	4.5
9	TN 200	0	0	0	2.75	3.0	3.5	4.75	4.25	4.25	5.5	4.5	5.75
11	Gregory	0	0	0	0	0	0	0	0	0	0	0	2.5
12	Travis	0	0	3.5	4.75	5.25	6.0	4.5	4.0	2.75	4.75	4.0	3.75
13	Del Carmen	0	0	3.5	4.25	4.5	5.25	5.5	4.75	5.25	4.75	5.5	5.5
14	Tow	0	0	0	0	0	2.0	2.5	3.5	5.5	5.25	3.75	4.75
15	CH 96-51	0	0	0	0	0	0	0	0	2.25	3.0	3.75	4.75
16	Harrington	0	0	0	0	0	0	0	0	0	2.0	2.75	3.0
17	CHHE-29	0	0	0	2.0	2.75	3.5	3.75	3.0	4.25	4.5	4.0	4.25
18	Metcalf	0	0	0	0	0	2.0	3.0	3.25	4.5	4.75	5.25	5.0
19	Burke	0	0	0	0	0	0	0	0	0	2.5	3.0	3.5
20	JV-1	0	0	0	0	0	2.5	3.0	3.5	3.0	3.5	3.0	3.25
21	Farina	0	0	0	0	0	0	0	0	0	2.25	2.25	2.75
22	Harris	0	0	0	0	2.5	2.0	2.5	3.75	4.75	5.5	5.75	5.75
23	Williamson	0	0	0	0	0	0	0	2.25	2.5	2.5	3.0	3.0
24	DeCamp	0	2.25	3.5	4.5	4.75	5.25	5.25	6.0	5.75	6.25	5.5	5.5
25	JV-4	0	0	0	0	0	0	2.0	2.5	2.75	3.5	3.75	4.0
26	Coronel	0	0	0	0	0	0	2.25	0	2.0	2.5	3.0	3.5
27	Bacon	0	0	0	0	0	0	0	0	2.0	2.5	2.5	2.75
29	JV-10	0	0	0	0	0	2.5	2.5	3.5	3.75	5.5	5.5	5.5
30	Bastianni	0	0	0	0	0	0	2.25	2.5	2.5	3.0	2.75	3.25
32	PR-10	0	0	2.25	0	2.0	2.5	3.5	3.25	3.5	4.5	5.25	5.0

0 = < 2

* = Number of fractions

** = NaCl molarity of elution fractions

corresponded to the phase S and B virions described by KARZON [5]. This observation suggested that the intratypic differences described for type 6 echoviruses would depend on the actual ratio of these two virions in a given

from DEAE cellulose column

fractions (log CPD ₅₀ /ml)											Titre of the standard virus
12 0.06	13 0.074	14 0.09	15 0.12	16 0.16	17 0.17	18 0.2	19 0.26	20 0.30	21 0.36	22 0.42	
5.75	5.5	4.75	5.25	4.0	4.25	4.5	3.0	3.25	3.0	3.25	7.0
3.75	4.25	4.0	4.5	5.0	4.75	5.5	5.75	6.25	6.0	6.0	6.75
5.5	4.75	4.25	5.0	6.25	4.25	4.5	4.5	4.25	3.75	3.25	6.5
6.0	5.75	5.0	4.75	4.0	4.25	3.75	4.0	4.0	4.25	4.25	6.0
0	2.75	4.0	5.5	5.5	6.0	6.0	5.5	5.0	5.5	5.5	6.25
4.25	4.25	4.5	6.75	6.5	6.25	5.25	5.5	4.75	4.25	4.5	7.5
4.5	4.25	5.25	2.5	2.75	4.55	4.45	4.0	3.75	3.0	3.25	7.25
3.25	3.5	2.75	2.5	2.0	0	2.0	0	0	0	0	6.75
5.5	6.0	6.25	6.75	5.25	4.5	4.5	3.25	3.5	3.25	3.0	6.75
5.75	6.25	6.25	7.75	6.5	6.25	5.25	4.5	4.5	4.25	4.5	8.25
3.5	4.25	4.5	5.5	5.75	5.0	4.75	4.5	3.25	3.25	3.0	5.75
3.25	3.0	3.25	2.75	2.5	2.75	2.0	0	0	0	0	6.25
6.0	5.75	5.75	5.5	4.5	4.5	5.5	5.5	5.5	5.5	4.5	6.5
4.25	3.75	3.0	3.5	3.0	3.25	2.75	3.0	3.0	2.75	2.5	5.75
4.5	5.5	5.75	6.0	6.25	5.25	5.25	6.0	5.75	5.75	5.5	6.5
3.75	5.0	4.75	5.25	5.25	5.75	6.0	4.75	5.75	5.75	5.25	6.5
4.5	4.25	4.5	5.75	5.25	5.5	4.75	4.0	4.5	4.25	3.75	6.0
5.25	4.75	5.25	5.75	5.5	5.75	6.0	5.5	5.5	5.5	5.25	6.25
4.75	5.25	4.5	6.25	6.0	5.5	4.75	3.5	3.5	3.0	3.0	6.5
3.75	4.75	5.75	5.0	4.75	4.0	4.25	4.25	3.75	3.0	3.0	6.0
3.0	3.5	4.0	4.5	4.25	5.5	5.25	4.75	5.0	4.5	4.25	5.75
6.25	6.0	4.75	4.0	3.25	3.0	2.75	2.5	2.25	2.0	2.5	6.5
3.25	3.75	3.75	4.25	4.5	4.75	5.5	5.75	5.25	4.75	4.25	6.0
5.75	5.5	5.5	4.5	4.25	4.5	4.5	4.75	4.5	4.5	3.75	6.5
4.75	4.5	5.5	5.75	5.5	4.75	4.5	4.25	4.0	4.0	3.75	6.0
3.5	4.0	4.0	4.5	5.0	5.5	4.0	3.75	3.5	3.0	3.0	5.75
3.25	3.5	3.5	3.75	4.25	4.0	4.25	4.5	5.75	5.5	5.5	6.0
4.5	4.5	4.25	4.5	4.5	3.25	3.25	3.0	2.0	0	0	6.25
3.5	3.75	4.75	5.5	5.5	5.0	5.75	6.0	6.45	5.75	5.5	6.5
5.0	5.75	6.5	6.0	6.25	6.0	5.5	5.75	5.0	5.25	5.0	6.75

population. Antigenic relationships between types 1, 8 and 12 have been detected by cross neutralization tests in several laboratories [13, 14, 15]. The discrepancies of cross-neutralization tests with identical strains originating from different sources suggested a variation of the ratio of virions of different antigenic characters in these strains.

Intratypic differences of enterovirus strains were also detected by chromatography by several authors [1, 2, 3, 16, 17, 18, 19].

The double elution peaks observed in the present study with prototype strains 1, 3, 7 and 12 suggested the possibility that a given virus strain may contain virions differing in one or another character similarly to those studied in detail by DÖMÖK and SIMON [20]. Two practically identical peaks were observed in the elution of prototype strain 1. With other strains, however, the two peaks differed by several orders of magnitude. The difference appeared to depend on the number of previous passages as well as on the origin of the given strain. Of the prototype strains 1 obtained from two different sources, one contained a major proportion of virions eluting at higher NaCl concentrations, while the other strain contained practically identical amounts of virions eluting at two different concentrations.

In their studies on type 19 echovirus, DÖMÖK and SIMON [20] succeeded in isolating 9 different mutants, using the H^+ , H^- , agar polysaccharide, and hydrolysed starch sensitivity markers. They have considered only one of the nine mutants to occur *in vivo*. Different conditions of cultivation [21] may favour either one or another of the mutants, resulting in considerable intratypic differences between the different strains maintained in different laboratories.

Accordingly, the results of chromatographic analysis presented in Table II are valid only for the strains propagated under the conditions outlined above and at the passage levels reported. The chromatographic homogeneity as well as the heterogeneity of the type strains maintained under standard conditions of passage were found to be reproducible. Chromatographic analysis of all prototype strains at three different passage levels yielded satisfactorily fitting results with all but 6 strains. In these cases the peaks were shifted within the range of 2–3 fractions. It is, however, difficult to ensure standard conditions of cultivation, as even minute differences in the individual monkey kidney cells may cause substantial shifts in the composition of the virion population.

The question therefore arises whether serologically uniform prototype strains maintained under different conditions of cultivation should really be considered identical. Identity in this particular case would mean identity of the surfaces' molecular structure, too.

There are few reliable data concerning the electrochemical structure of the virion's surface [22]. Suggestions on the groups possibly involved in the adsorption of a given virion to a given adsorbent are also scarce. THOMSEN [23] supposed the involvement of free carboxyl groups in the neighbourhood of OH groups in the adsorption of poliovirus to $Al(OH)_3$ gel. In spite of the remarkable studies of THOMSEN [24] on the adsorption of ^{32}P labelled Mahoney and LSc 2ab poliovirus strains before and after the reaction with antibody, information is still lacking on the possible relations between the active surface

groups responsible for the adsorption to a given adsorbent and those determining the antigenic character of a given virion.

The data presented seem to be helpful in stimulating further, more detailed studies on the fine antigenic and molecular structure of the virions-surface and a more detailed characterization of prototype echovirus strains.

Acknowledgement. The authors are indebted to Dr. R. THOMSEN (Hygiene-Institut de Albert Ludwig Universität, Freiburg i. Br., Germany) for valuable help in initiating the study to Dr. I. DÖMÖK (National Institute of Public Health, Budapest) for the prototype strains and sera, and to Miss E. LÁSZLAI for excellent technical assistance.

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REPLICATION OF HERPES SIMPLEX VIRUS IN ARGININE-FREE MEDIA

I. EFFECT OF ARGININE-DEFICIENCY IN DIFFERENT TISSUE CULTURE CELLS

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(Received September 22, 1966)

Summary. The replication of *Herpes simplex* virus (HSV) in arginine-free media is inhibited in HeLa, HEP₂ and KB cells, while in human embryonic fibroblasts, chick embryo fibroblasts, and primary monkey kidney cells virus replication is unimpaired.

The absence of arginine from the medium does not affect the adsorption of HSV to HeLa or human embryonic fibroblast cells nor the release of virus from the same cells. The absence of arginine seems to inhibit some event of viral replication after the adsorption (penetration, reproduction). The lack of arginine inhibits the development of typical cytopathic effect as well as the appearance of specific viral antigens in HeLa cells.

LEWIS and SCOTT [1] have shown that glutaminic acid is indispensable for the replication of *Herpes simplex* virus (HSV) in HeLa cells. TANKERSLEY [2] performed comparative studies on the action of the 13 amino acids incorporated into the Eagle medium on the replication of HSV in cells of a human oesophageal cell line. With the exception of lysine, all amino acids and especially histidine and arginine were necessary for optimal virus growth. Arginine appeared to have a particularly important role as in arginine deficient media a phenomenon resembling latent virus infection occurred. Similar results were obtained by SHARON [3], who observed that satisfactory replication of HSV in Wish cells depended on the presence of adequate amounts of arginine.

The present study was performed to examine the inhibitory effect of arginine deficiency on the replication of HSV isolated in this laboratory and propagated in different cell cultures, and also to gain some information on the mechanism of the inhibitory action.

Materials and methods

Virus. The HSV strain used in the present study was isolated from vesicle contents obtained from a patient with genital herpes in 1962. The strain exhibited cytopathic effect in HeLa and human embryonic fibroblast cells. The effect was of the mixed type described by HOGGAN and ROIZMAN [4]. The average virus titre obtained in HeLa cells was $10^{6.5}$ TCD₅₀/ml.

Tissue culture. The virus was maintained by serial transfers in HeLa or human embryonic fibroblast cells. Fibroblast cells were cultured as described previously [5]. Growth curves of the virus were obtained in PPLO-free HeLa, HEP₂ and KB cultures. PPLO was eliminated from these cultures by periodic treatment with kanamycin (Resistomycin Bayer, 1 g = 1.44 g kanamycin sulphate) [6]. Human embryonic fibroblast, primary monkey kidney and primary

chick embryo fibroblast cells were used without treatment. The efficiency of kanamycin treatment was checked by BARILE's arginase test [7]. The three cell lines failed to exhibit arginase activity at the time of the experiments.

Media. Parker's No. 199 medium containing 10 and 20 per cent bovine serum was used for the cultivation of HeLa, HEp₂, KB, and human embryonic and chick embryonic fibroblast cells, respectively. Primary monkey kidney cells were cultured in Hanks' solution containing 5 per cent lactalbumin hydrolysate and 2 per cent bovine serum. For maintenance, serum-free Parker's 199 medium was used with all cell types.

Growth curves of HSV. The cell monolayers grown in tubes were washed with PBS. Inoculation was performed at a multiplicity of 4 and adsorption was allowed to take place for one hour at 37 °C. This was followed by three consecutive washes with PBS and the cultures were given either arginine free (A⁻) or complete (A⁺) Parker's 199 solution. The inoculated cultures were incubated at 37 °C. Samples were taken at appropriate intervals and titrated in 10 parallel tube cultures. With HeLa and human embryonic fibroblast cells, the samples were taken 1, 2, 3, 4, 6, 8, 12, 17, 25 and 33 hours after the infection and the extra- and intracellular virus was determined separately. For the quantitation of intracellular virus the cells were scraped off into fresh arginine-free Parker's 199 medium, disintegrated by sonication and centrifuged. The supernatant was considered to represent a suspension of intracellular virus. With HEp₂, KB, primary monkey kidney and chick embryo fibroblast cells, the samples were taken 6, 12, 24 and 48 hours after the infection. In these samples, only the total virus content was determined. All infectivity titrations were performed on human embryonic fibroblast cells. Titrations were read on the 2nd, 4th and 6th days. TCID₅₀ was computed according to REED and MUENCH, from the results read on the 4th day [8].

Adsorption experiment. Flask cultures of HeLa cells infected with HSV were washed 3 times with PBS. The cells were scraped off by means of a glass rod and suspended in arginine-free Parker's medium. After disintegration by sonication and centrifugation, the supernatant served as stock virus suspension ("arginine-free virus suspension"). Human embryonic fibroblast and HeLa cell culture tubes were inoculated with a tenfold dilution series of this virus. The maintenance medium of infected cultures was either A⁻ or A⁺ Parker's medium. Adsorption took place at 37 °C. Four tubes each were washed 3 times with PBS and overlaid with A⁺ Parker's 199 after 10, 20, 30, 60 minutes or 18 hours following the addition of virus. The cultures were then returned to the incubator (37 °C) and the effectiveness of adsorption was determined in terms of the titres obtained on the 4th day.

Immunofluorescent technique. The indirect technique described by KOLLER *et al.* [9] was used. Convalescent patient serum served as immune serum.

Results

Replication of HSV in different primary and tumour cells maintained in arginine-free medium. Fig. 1 presents the growth curves of HSV in different primary cell cultures and in cells of different human tumour cell lines. In human embryonic fibroblast, primary monkey kidney and chick embryo fibroblast cells, arginine deficiency failed to influence viral replication. In contrast, virus replication was inhibited in HeLa, HEp₂ and KB cells maintained in arginine-deficient medium.

For a more detailed study of the virus replication inhibitory effect of arginine deficiency, HeLa and primary human embryonic fibroblast cells were selected.

Adsorption of HSV to HeLa and human embryonic fibroblast cells in arginine-deficient media. Results are presented in Table I. No increase was demonstrable in the amount of adsorbed virus after 20 minutes. Arginine deficiency did not influence the adsorption either to HeLa or to human embryonic fibroblast cells.

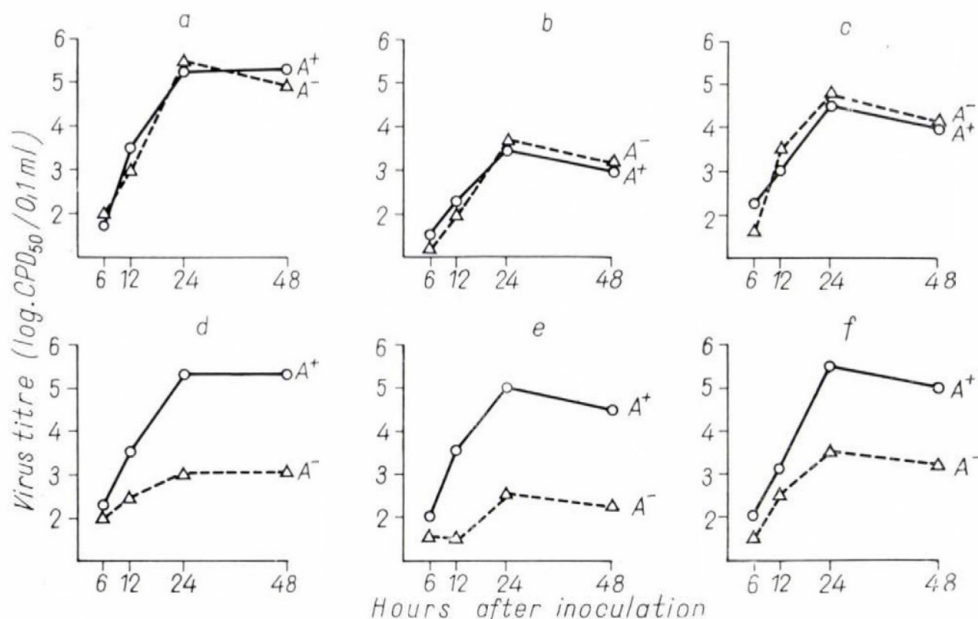


Fig. 1. Growth curves of *Herpes simplex* virus in complete (A⁺) and arginine-free (A⁻) media in human embryonic fibroblast (a), primary monkey kidney (b), primary chick embryo fibroblast (c), HeLa (d), HEP₂ (e) and KB (f) cells

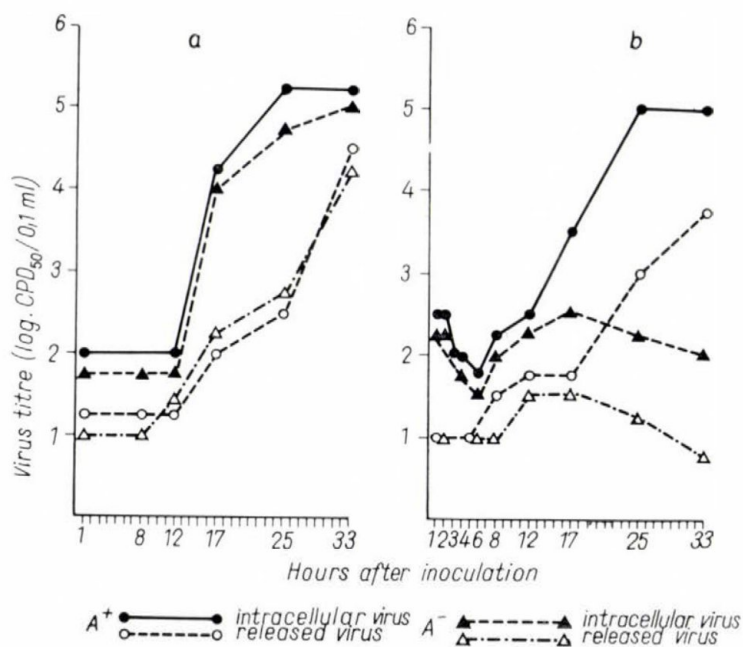


Fig. 2. Replication of *Herpes simplex* virus in human embryonic fibroblast (a) and HeLa (b) cell cultures in complete (A⁺) and arginine-free (A⁻) media

Table I

Adsorption of Herpes simplex virus to human embryonic fibroblast and HeLa cells in arginine-free (A^-) and complete (A^+) medium

Cell culture	Medium during adsorption	Virus titres (log CPD ₅₀ /0.1 ml) after adsorption for					No exchange of medium
		10	20	30	60	1048	
		minutes					
Human embryonic fibroblast	A ⁻	4.25	5.50	5.75	5.75	5.50	5.50
	A ⁺	3.75	5.25	5.50	5.50	5.75	5.25
HeLa	A ⁻	4.25	5.50	5.50	5.25	4.75	2.50
	A ⁺	4.25	5.50	5.50	5.75	5.50	5.50

Comparison of the amount of intra- and extracellular virus in human embryonic fibroblast and HeLa cells maintained in arginine-deficient and complete media. As revealed by Fig. 2, the virus growth curves in human embryonic fibroblast cells were identical in both arginine-deficient and complete media. No change in the amount of virus was observed on deficient medium, — either in intracellular or in extracellular virus yields. Growth curves obtained in HeLa cells, however, differed considerably in the two types of medium. The amount of intracellular virus was identical until the 12th hour in both arginine-deficient and complete medium. Later the titres obtained in arginine-deficient medium tended to decrease consistently in comparison to those in the control medium. In the 25th hour, this difference attained 2.75 logs. The extracellular virion appeared also in arginine-deficient medium and up to the 17th hour the titres were equally low in both arginine-deficient and control media, when a conspicuous divergence appeared and in the complete medium the curve ascended steeply where as in the arginine-deficient medium it stagnated or showed a decrease. The titre difference amounted to 1.75 log in the 25th hour.

Table II

Development of cytopathic effect and appearance of virus antigen in Herpes simplex virus infected HeLa cells maintained in arginine-free (A^-) and complete (A^+) media

Medium	Highest virus dilution -log ₁₀ giving positive reaction	
	Unstained	Immunofluorescence
A^+	5	5
A^-	2	2
A^-, A^+ after 24 hours	4	4
A^-, A^+ after 48 hours	3	3
A^-, A^+ after 72 hours	2	2

Cytopathic effect and specific immunofluorescence in HeLa cells maintained in arginine-deficient medium. Monolayers of HeLa cells grown on coverslips or in tubes were inoculated with a tenfold dilution series of HSV. After adsorption and three subsequent washes, the cultures received arginine-free or complete medium and were then incubated at 37 °C. Titres were read 24 hours after the infection in cultures maintained in arginine-deficient medium. In cases when the arginine deficiency had been suspended by the addition of complete medium, evaluation took place 24 hours after completion of the medium. The cultures were examined either unstained or by immunofluorescence.

A titre decrease of 3 logs was demonstrable by both methods in infected cells maintained in arginine-deficient media. The difference was reduced to 1 log when arginine deficiency had been suspended after 24 hours. The later it had been suspended (48, 72 hours), the less cytopathic changes occurred in cultures infected with higher virus dilutions. Identical results were obtained with immunofluorescence.

Discussion

There are several data available in the literature on the importance of arginine in uninfected as well as virus infected tissue cultures. THOMAS *et al.* [10] have shown that with L, HeLa and ERK cell lines the repeated addition of arginine to the medium ensured results equivalent to those obtained after complete medium substitution. Other authors [11, 12, 13] were able to influence the replication of adenoviruses by changing the arginine concentration in the medium.

It has been known that cell cultures are frequently infected with PPLO which organism specifically reduces the medium's arginine contents [12, 14]. Therefore, we had to ensure the absence of PPLO from the cultures used by periodical kanamycin treatment. The absence of PPLO was checked by the determination of arginase activity. As arginine deficiency had no influence on virus replication in the primary cells, these were not treated with kanamycin, nor was their PPLO-contamination examined.

Similarly to the observations reported by TANKERSLEY [2] and SHARON [3], we, too, have observed the virus replication inhibitory action of arginine deficiency in HeLa, HEp₂ and KB cells. We failed, however, to demonstrate a similar effect in human embryonic fibroblasts, chick embryo fibroblast and primary monkey kidney cells.

It has been known for other viruses that the requirement of a given amino acid depended largely on the nature of cell cultures used in the study. The replication of poliovirus in HeLa cells requires the presence of salts, glucose and glutamine [15]. In primary monkey kidney cells, in addition to salts only the presence of L-cysteine is essential [16, 17]. According to TYNDALL [18],

poliovirus replication in permanent monkey kidney cells greatly depends on the L-cysteine content of the medium. No similar requirement of the presence of L-cysteine was, however, observed for the replication of poliovirus in permanent rabbit kidney and HeLa cells. The phenomenon is as a rule explained by quantitative differences in the free amino acid content of the different cells. The phenomenon observed by us may have some similar causes, primary cell cultures having probably higher arginine stores than permanent cell lines, thus offering favourable conditions for virus replication even in arginine deficient media.

The fact that in the present experiments only the tumour cells exhibited a definite arginine requirement, suggests the possibility that their enhanced metabolism may be the cause underlying the increased amino acid requirement, particularly in cells infected with HSV. This would explain why virus replication is poorer in such cells in arginine-deficient media. This possibility has been supported by reports on an increased incorporation of arginine and other amino acids into the histones and other nuclear proteins of tumour cells as compared to normal ones [19, 20].

Another explanation of the phenomenon might be that primary cells are more capable of overcoming arginine deficiency than tumour cells.

Examination of different phases of the viral replication cycle in HeLa cells has shown that arginine deficiency inhibits neither the adsorption nor the release of the virus, since the amount of free and cell-bound viruses was always proportional. It has therefore been concluded that arginine deficiency inhibited some other phase of virus reproduction (penetration, replication).

In cells maintained in arginine-deficient medium, both the development of cytopathic effect and the appearance of virus antigen demonstrable by immunofluorescence were uniformly inhibited. In cultures infected with low amounts of virus and maintained in arginine deficient medium, neither cytopathic effect nor specific immunofluorescence developed. On the addition of arginine, however, both methods revealed changes typical of virus replication. This, however, depended on the inoculum and the time of arginine addition, thus the presence of a true latent virus carriership was not probable. Maintenance of infected HeLa cells in arginine deficient medium resulted in a phenomenon similar to the latent virus infection described by TANKERSLEY [2]. However, in the former case neither cytopathic effect nor the appearance of virus antigen was demonstrable in spite of the presence of the virus in the cells.

Acknowledgements. The excellent technical assistance of Mrs. K. DÓCZY is duly acknowledged.

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REPLICATION OF HERPES SIMPLEX VIRUS IN ARGININE-FREE MEDIA

II. DNA SYNTHESIS IN INFECTED AND NON-INFECTED HUMAN EMBRYONIC FIBROBLAST AND HELA TISSUE CULTURE CELLS

By

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(Received September 22, 1966)

Summary. In arginine-free medium, HeLa cells exhibit a lower rate of metabolism, DNA synthesis being particularly impaired. In human embryonic fibroblast cells, the absence of arginine did not cause measurable change in metabolism, as estimated by ^{32}P incorporation. In arginine-containing media, HeLa cells infected with *Herpes simplex* virus exhibited an enhanced ^{32}P incorporation rate into DNA. In the absence of arginine, there was no difference between infected and non-infected cells.

As measured by the Burton method, the total DNA content of cells did not change after infection with HSV or in the absence of arginine.

The rate of ^{32}P incorporation into HeLa and human embryonic fibroblast cells has been examined in arginine-deficient media, and also into HeLa cells infected and not infected with *Herpes simplex* virus (HSV) maintained in arginine-free and complete media. The aim was to elucidate whether the increase of DNA synthesis connected with virus replication [1] occurred in arginine-free media similarly as in the control medium, and to clarify whether the phenomenon similar to latent virus infection observed in arginine-free media would induce any sign of enhanced DNA synthesis.

Materials and methods

Virus. The strain used has been specified previously [2].

Tissue cultures. Kanamycin-treated HeLa and untreated human embryonic fibroblast cells were used throughout. Fibroblast cultures were prepared as described earlier [3].

Media. The growth medium was Parker's No. 199, containing 10 and 20 per cent bovine serum for HeLa and human embryonic cells, respectively. Maintenance medium was Parker's 199. Experimental media were arginine-free and complete Parker's 199 containing 10 μC activity in $\text{Na}_2\text{H}^{32}\text{PO}_4/\text{ml}$.

Experimental procedure. Seven days old HeLa and human embryonic fibroblast cultures were washed twice with PBS, infected with 4 CPD₅₀/cell HSV. Adsorption was allowed for 1 hour at 37 °C. Uninfected cultures underwent the same treatment, using Parker's 199 medium instead of virus. The infected cultures were repeatedly washed and overlaid with the experimental medium. The cells were fractionated according to SCHMIDT—THANNHAUSER [4] after 15 or 24 hours incubation at 37 °C. ^{32}P activity was determined in the lipid, RNA and DNA fractions. For the activity measurements (counts/min.) an Orion EMG 1872 decimal scaler was used.

The DNA content of DNA fractions was determined by the diphenylamine method of DISCHE as modified by BURTON [5]. Calf thymus DNA was used as reference standard.

All analytical data were calculated for 1 mg dry substance; this was determined by drying a known volume of cell suspension by means of an infra-red lamp and weighing the dry substance by an analytical balance.

Results

Specific activity of lipid, RNA and DNA fractions of uninfected human embryonic fibroblasts and HeLa cells maintained in arginine-free and complete media. The ^{32}P activity of lipid, RNA and DNA fractions of uninfected human embryonic fibroblast cells was identical in both arginine-free and complete media. In contrast, the activity of lipid and RNA fractions of HeLa cells decreased moderately (by 16–18 per cent), and that of the DNA fraction considerably (by 61 per cent) when the cells had been maintained in arginine-free medium for 24 hours prior to fractionation (Table I).

Table I

*Specific activity of human embryonic fibroblasts and HeLa cells in complete (A^+) and incomplete (A^-) media**

Cell culture	Medium	Lipid		RNA		DNA		Total ^{32}P	
		Counts/min.**	%	Counts/min.	%	Counts/min.	%	Counts/min.	%
Human embryonic fibroblast	A^+	8 790	100	11 469	100	2 011	100	49 941	100
	A^-	9 229	105	11 222	98	2 071	103	50 862	102
HeLa	A^+	10 920	100	22 493	100	8 500	100	67 445	100
	A^-	9 172	84	18 550	82	3 315	39	61 398	91

* = Flask cultures were maintained in the experimental media for 24 hours prior to fractionation

** = ^{32}P activity/1 mg dry material

DNA content and specific activity of DNA fractions of infected and uninfected HeLa cells maintained in arginine-free and complete media. As shown in Table II, the specific activity of DNA fraction of HeLa cells in complete medium exhibited a twofold increase 15 hours after the infection, as compared to the activity of the control cells. The same difference was demonstrable in the 24th hour. In arginine-free medium, however, no notable increase in the activity of the DNA fraction of infected HeLa cells was observed at 15 and 24 hours.

In this experiment as well as in that demonstrated in Table I, the activity of DNA of HeLa cells was considerably lower in arginine-free than in the complete medium. The difference amounted to 50 and 60 per cent in the 15th and 24th hours, respectively.

Table II

DNA content and ^{32}P activity of DNA fractions of HSV infected and uninfected HeLa cells maintained in complete (A^+) and arginine-free (A^-) media

Medium	Time of fractionation (hours)	Uninfected cells		HSV-infected cells	
		$^{32}\text{P}^*$	DNA**	^{32}P	DNA
A^+	15	6280	21.5	12 800	21.8
	24	8540	22	15 640	22.3
A^-	15	3160	21.6	3 520	21.5
	24	3415	21.9	3 315	22.1

* = Counts/min./1 mg dry material

** = $\mu\text{g}/1$ mg dry material

No significant difference was demonstrable in the total DNA content of HeLa cells infected or uninfected and maintained either in arginine-free or in complete media.

Discussion

We have shown that the virus replication impairment caused by the absence of arginine fails to appear if HSV is grown in primary human embryonic fibroblasts, primary monkey kidney or primary chick embryo fibroblast cells. A clear-cut effect was, however, demonstrable if HeLa, HEP₂, or KB cells were used [2]. Thus, we supposed that the inhibitory action of arginine deficiency might have its cause in some factors of cellular metabolism.

The use of arginine-free medium did not cause any microscopically apparent morphological changes of the cells. Live cell counts determined by means of trypan blue vital staining have shown that the ratio of live to dead cells was identical in both arginine-deficient and complete media [6]. The rate of DNA synthesis computed from the ^{32}P -incorporation by HeLa cells was considerably lower in arginine-deficient than in complete medium. Similar results have been reported by ACKERMANN [7]. It should, however, be noted, that in the present experiment arginine deficiency impaired only the metabolism of HeLa cells, thus the cause of virus reproduction inhibition seemed to be the result of such an impairment [2].

Human embryonic fibroblast cells produced virus normally in arginine-deficient medium and suffered no impairment in ^{32}P incorporation either. Therefore, the further studies were performed only on HeLa cells infected with HSV and maintained in arginine-deficient or complete medium.

Increased rate of DNA synthesis was demonstrable by increased incorporation of labelled phosphorus and thymidine in cells infected with *Herpes simplex*, pseudo-rabies or adenoviruses [1, 8, 9]. In our experiments, a higher ^{32}P incorporation into the DNA fraction of HSV-infected HeLa cells was demonstrable as compared to the incorporation by uninfected cells (see Table II).

HeLa cells infected with HSV and maintained in arginine-free medium exhibited a low ^{32}P incorporation into their DNA fractions, similarly to uninfected cells. Thus, the enhanced incorporation rate characteristic of virus infection failed to appear in arginine-free medium.

The chemically demonstrable DNA content of HeLa cells was practically identical whether they were infected or not. This observation contradicts those by NEWTON and STOKER [10] and WILDY *et al.* [11] who found an increase in the amount of DNA as a result of HSV infection. On the other hand, our results agree well with those of RUSSEL *et al.* [1] who found the total DNA content of HSV-infected BHK 21 cells unchanged throughout the viral replication cycle.

We did not find any difference in the DNA contents of uninfected HeLa cells maintained in arginine-free or complete medium. The DNA content of HeLa cells was found to be $15 \mu\text{g}/10^6$ cells, very similar to the data by NEWTON and STOKER [10] and LESLIE *et al.* [12].

As to the mechanism of the virus replication inhibitory action of arginine deficiency, no conclusive evidence has been obtained in the present experiments. Nevertheless, the decreased ^{32}P incorporation rate suggested that in the absence of arginine there was a decrease in the rate of metabolism of HeLa cells, particularly in that of their DNA. Thus, we suppose that the inhibition of virus replication is part of the general inhibition of cell metabolism. This seems to be supported by the fact that the absence of arginine caused neither an impairment of metabolism nor an inhibition of virus replication in primary human embryonic fibroblast cells.

Acknowledgements. We are indebted to Dr. A. ZSINDELY, Institute of Biochemistry, University Medical School, Debrecen, for calf thymus DNA. The excellent technical assistance of Miss É. MIKÓ is duly acknowledged.

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RELATION OF THE ANTIBACTERIAL ACTIVITY AND CHEMICAL STRUCTURE OF CHALCONE DERIVATIVES*

By

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(Received October 10, 1966)

Summary. The antibacterial activity of nitrohydroxychalcone derivatives on *Staphylococcus albus* and *aureus* was studied by the agar plate method.

Optimal bacteriostatic effect was observed with a structure containing the hydroxyl group in position 4'. Derivatives with a hydroxyl in position 3' or 2' were less active. As compared to the hydroxyl group, the nitro group had a lesser, though favourable effect on activity. Activity was enhanced by halogenic substituents in positions 2 and 4. In this respect, fluorine was superior to bromine or chlorine. Chalcones obtained with naphthaldehyde, p-isopropylbenzaldehyde or piperonal were often active, those obtained from 3-nitro-salicylaldehyde always active.

Chalcone derivatives have been known to have several pharmacological activities such as cholagogic, spasmolytic, laxative, etc. effects [1, 2, 4]. Their antibacterial activity has been scarcely examined. In the following we shall report on studies of several, mainly nitro-hydroxy-chalcone derivatives; to our best knowledge, their antibacterial effect has not been investigated. Our further aim was to collect some data on the possible relations of the chemical structure and antibacterial activity of chalcone derivatives.

Materials and methods

Antibacterial activity was examined by the agar plate method. 0.5 ml of a tenfold dilution of a 16-hour broth culture of the bacterium was added to agar and poured into a petri dish. Holes 12 mm in diameter were cut into the solidified plates. In each well was placed 0.1 ml of different dilutions of the test substance. Results shown in the Tables refer to the amount of test material contained in 0.1 ml. The cultures were incubated for 16 hours at 37 °C. Diameters of inhibition zones were measured with a vernier.

Chalcone derivatives were diluted in ethanol-water mixture (the lowest possible ethanol concentration was always used, depending on the solubility of the given derivative). No inhibitory effect was observed with the ethanol concentrations used.

At the beginning, the antibacterial effect was tested on *E. coli*, *Proteus vulgaris*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Candida albicans*, *Bacillus subtilis* and 6 different strains of *Staphylococcus*.

Antibiotic sensitivity of the different *Staphylococci* was determined with the Biotest disc set.

* Part of this paper was presented at the Bioflavonoid Symposium organized by the University Medical School, Szeged and the Committee on Flavonoids of the Hungarian Academy of Sciences, May 26, 1965, in Szeged.

Staphylococcus albus: sensitive to erythromycin; moderately sensitive to aureomycin, terramycin, tetracycline, chloramphenicol and neomycin; resistant to penicillin, streptomycin, polymyxin.

Staphylococcus aureus: sensitive to aureomycin, terramycin, tetracycline, erythromycin; moderately sensitive to penicillin, streptomycin, chloramphenicol and neomycin; resistant to polymyxin.

Diameters of the inhibition zones of different amounts of some antibiotics as measured by the agar plate method, are given in Table I.

Table I
Inhibition zones of different amounts of some antibiotics

Amount of antibiotics		Diameter of inhibition zone, mm	
		<i>Staph. albus</i>	<i>Staph. aureus</i>
Penicillin	25 μ g	22.0	31.0
	5 μ g	14.0	27.0
Chloramphenicol	25 μ g	19.5	19.5
	5 μ g	—	—
Neomycin	25 μ g	18.0	17.0
	5 μ g	15.0	15.0

Results and discussion

None of the chalcone derivatives had any effect even at concentrations exceeding 500 μ g on the organisms tested, except for *Bacillus subtilis* and the 6 different strains of *Staphylococci*. Thus, all other microorganisms were excluded from the further studies. The different strains of *Staphylococci* exhibited different sensitivities to the various chalcone derivatives. Antibacterial activity of the chalcone derivatives was measured also by other authors on *Staphylococci* as test organisms, thus these organisms have been selected for testing in the present experiment. We have examined a total of 97 chalcone derivatives. Out of them, 88 were nitrohydroxy, 3 nitrohydroxy-bromine, 2 nitro- and 4 hydroxy derivatives. Results are shown in Table II.

The greatest activity was observed with compounds Nos. 3, 10, 14, 18, 19, 22, 26, 35, 36, 38, 53, 55, 56, 62, 63, 64, 65. The majority of the active compounds (11) belonged to the 3'-nitro-4'-hydroxy derivatives.

The bacteriostatic effect of aminochalcones has been reported by MARRIAN *et al.* [3]. The greatest effect was found with 4,4'-diaminochalcone. SCHRAUF-STÄTTER and DEUTSCH [5] examined the bacteriostatic effect on *Staphylococcus aureus* of different natural and synthetic chalcones. Admitting that no satisfactory evidence was available on the relation of the structure to bacteriostatic activity, these authors arrived at the following conclusions. (1) The most active

compounds were derivatives of 2-hydroxy chalcones (the highest activity was observed with 2,2'-hydroxy-3,5,5'-tribromochalcones). (2) The bacteriostatic effect was considerably higher with the substituents in the benzene ring deriving from the benzaldehyde and not in that of the acetophenone portion. (3) The presence of more than two hydroxyl groups lowered the activity.

SCHRAUFSTÄTTER and DEUTSCH obtained further conclusions in a subsequent study [5]. (1) Hydration of the double bond resulted in a loss of the bacteriostatic effect. (2) Reduction of the keto-group to alcohol lowered the activity. (3) The $-\text{CO}-\text{CH}:\text{CH}-$ group of the chalcone structure was less important, nevertheless substitution with a methyl or methoxy group in α position ($-\text{CO}-\text{C}:\text{C}-$) abolished antibacterial activity. (4) Of the halogen derivatives, α and β bromochalcones had considerable antibacterial activities.

The nitrohydroxy-chalcones used in our study were obtained from four different nitrohydroxyketones. These are shown in Fig. 1.

Studies were also performed on chalcone derivatives of the ketones listed in Fig. 2.

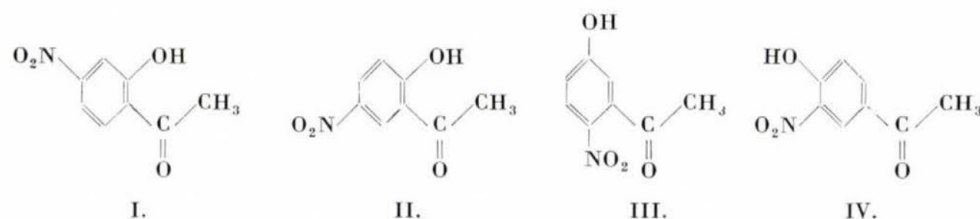


Fig. 1. Nitrohydroxy-ketones

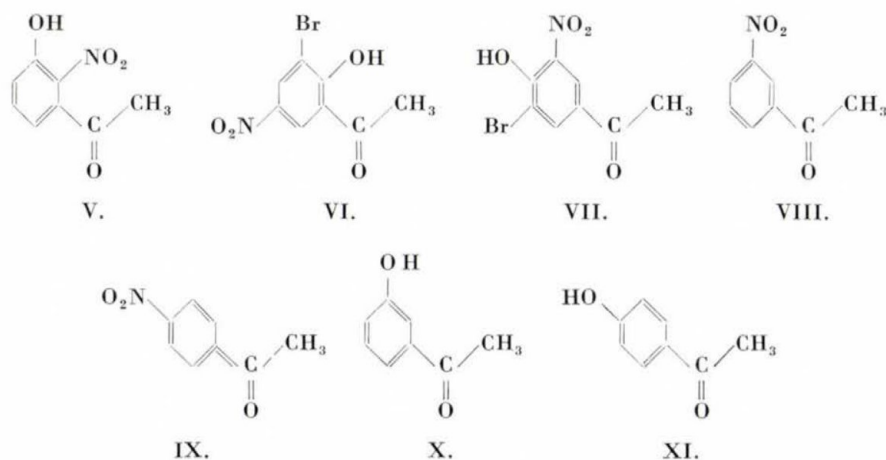
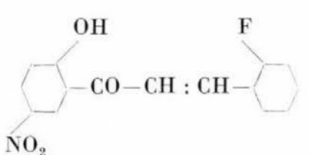
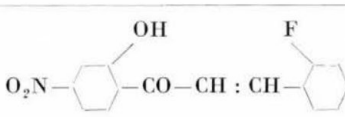
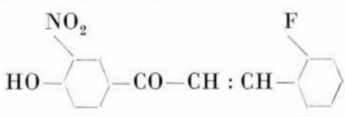
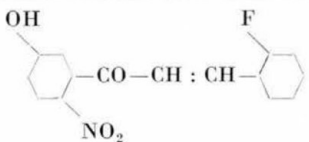
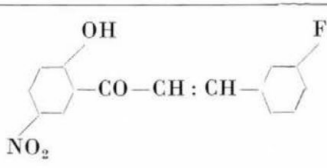


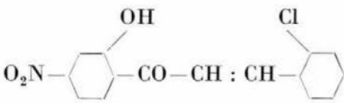
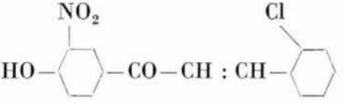
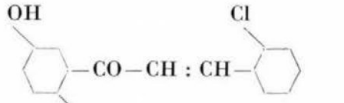
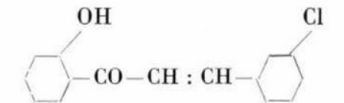
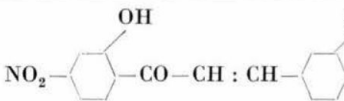
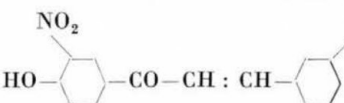
Fig. 2. Nitro-, nitrohydroxy-, nitrohydroxybromine- and hydroxyketones

Table II
Antibacterial Activity of Chalcone Derivatives

No.	Structural formula	Concentration, $\mu\text{g}/0.1 \text{ ml}$			Diameter of inhibition zone, mm					
					<i>Staph. albus</i>			<i>Staph. aureus</i>		
1.		50	25	12.5	21.0	25.0	23.0	14.0	—	—
2.		100	10	5	—	—	—	—	—	—
3.		33.3	16.6	8.3	31.2	28.3	24.2	24.0	22.0*	19.0*
4.		100	10	5	24.5	—	—	22.0	—	—
5.		50	25	12.5	—	—	—	—	—	—

6.		50	25	12.5	—	—	—	—	—	—
7.		100	10	5	30.0**	14.0**	—	26.0*	—	—
8.		33.3	16.6	8.3	—	—	—	—	—	—
9.		50	25	12.5	14.0	—	—	—	—	—
10.		100	10	5	20.0**	—	—	14.0	—	—
11.		50	25	12.5	30.0	28.0	26.0	24.5*	23.0*	18.0*
12.		33.3	16.6	8.3	14.0	—	—	15.5	—	—

Table II continued

No.	Structural formula	Concentration, $\mu\text{g}/0.1 \text{ ml}$			Diameter of inhibition zone, mm					
					<i>Staph. albus</i>			<i>Staph. aureus</i>		
13.		50	25	12.5	19.0*	16.5**	—	—	—	—
14.		50	25	12.5	32.5	30.0	27.0	27.5	25.5	23.0
15.		100	10	5	30.0*	21.0**	—	27.5	19.5	—
16.		50	25	12.5	14.0	—	—	—	—	—
17.		33.3	16.6	8.3	—	—	—	—	—	—
18.		50	25	12.5	38.5	35.0	33.0	28.7	28.0	25.0

19.		100	10	5	27.0**	25.0**	14.0**	27.0*	20.0*	14.0*
20.		50	25	12.5	—	—	—	—	—	—
21.		50	25	12.5	—	—	—	—	—	—
22.		100	10	5	33.2	28.3	24.5	30.0*	27.0*	22.3*
23.		100	10	5	23.0	20.0*	18.0*	21.0**	14.0*	—
24.		33.3	16.6	8.3	—	—	—	—	—	—

Table II continued

No.	Structural formula	Concentration, $\mu\text{g}/0.1 \text{ ml}$			Diameter of inhibition zone, mm					
					<i>Staph. albus</i>			<i>Staph. aureus</i>		
25.		33.3	16.6	8.3	—	—	—	—	—	—
26.		100	10	5	37.5	34.0	27.0	27.0	25.0	21.0
27.		50	25	12.5	—	—	—	—	—	—
28.		50	25	12.5	—	—	—	—	—	—
29.		50	25	12.5	21.5	19.6	19.0	20.6	19.2	16.5*
30.		100	10	5	21.3	19.0	16.5	14.0	—	—

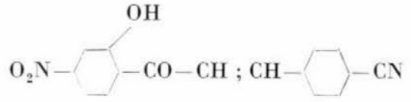
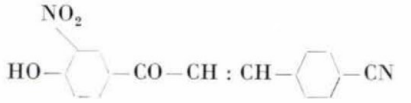
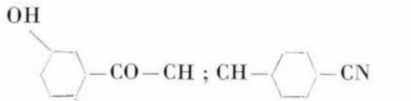
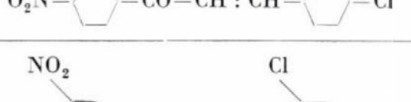
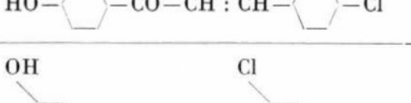
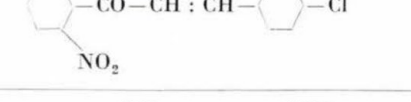
31.		50	25	12.5	—	—	—	—	—	—
32.		33.3	16.6	8.3	26.6	22.5	20.0	20.0*	14.0*	—
33.		100	10	5	27.0*	19.9*	—	28.3*	—	—
34.		50	25	12.5	—	—	—	—	—	—
35.		100	10	5	30.0	29.0	26.0	29.3*	26.0*	22.3*
36.		100	10	5	34.0	29.5	25.5	27.5	25.5*	21.0*
37.		33.3	16.6	8.3	14.0	—	—	—	—	—

Table II continued

No.	Structural formula	Concentration, $\mu\text{g}/0.1 \text{ ml}$			Diameter of inhibition zone, mm					
					<i>Staph. albus</i>			<i>Staph. aureus</i>		
38.		100	10	5	38.0	34.0	29.0	21.0	20.0	18.0
39.		100	10	5	25.0*	14.0*	—	26.0*	14.0*	—
40.		33.3	16.6	8.3	—	—	—	—	—	—
41.		50	25.	12.5	14.0	—	—	—	—	—
42.		50	25	12.5	—	—	—	—	—	—
43.		100	10	5	20.0*	—	—	24.5*	—	—

44.		100	10	5	—	—	—	—	—	—
45.		100	10	5	28.8	19.0	—	27.0*	19.2*	—
46.		100	10	5	19.5*	—	—	18.0**	—	—
47.		50	25	12.5	—	—	—	—	—	—
48.		50	25	12.5	—	—	—	—	—	—
49.		100	10	5	21.5	—	—	23.5	21.3	16.5
50.		100	10	5	—	—	—	—	—	—

Table II continued

No.	Structural formula	Concentration, $\mu\text{g}/0.1 \text{ ml}$			Diameter of inhibition zone, mm					
					<i>Staph. albus</i>			<i>Staph. aureus</i>		
51.		100	10	5	—	—	—	—	—	—
52.		100	10	5	—	—	—	—	—	—
53.		100	10	5	cr. p. 26.8	26.0	23.0	cr. p. 24.2	25.5	23.0
54.		33.3	16.6	8.3	—	—	—	—	—	—
55.		50	25	12.5	35.0	28.0	26.0	23.5	22.5	18.5**
56.		50	25	12.5	—	—	—	—	—	—

57.		100	10	5	24.5*	14.0	—	23.2*	16.0**	—
58.		33.3	16.6	8.3	—	—	—	—	—	—
59.		50	25	12.5	—	—	—	—	—	—
60.		50	25	12.5	cr. p. 25.0	23.0	21.2	14.0	—	—
61.		100	10	5	cr.p. 21.0	26.0	21.5	26.0**	24.0*	17.0*
62.		33.3	16.6	8.3	26.0	24.0	21.5	20.0	18.0	17.0
63.		33.3	16.6	8.3	34.0	30.0	29.0	32.6	30.0	24.0

Table II continued

No.	Structural formula	Concentration, μg/0.1 ml			Diameter of inhibition zone, mm					
					<i>Staph. albus</i>			<i>Staph. aureus</i>		
64.		100	10	5	38.5	33.0	29.0	22.5**	14.0**	—
65.		50	25	12.5	27.0*	25.5*	24.5*	26.0	24.2	23.5
66.		33.3	16.6	8.3	21.5	18.0	14.0	18.0	16.0	14.5
67.		100	10	5	—	—	—	—	—	—
68.		150	15	7.5	25.0**	22.0*	—	—	—	—
69.		250	25	12.5	30.0	19.0*	—	—	—	—

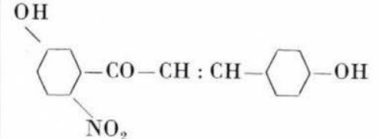
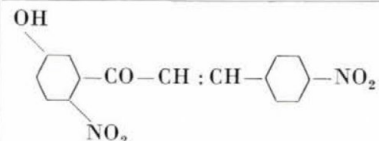
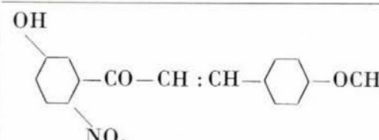
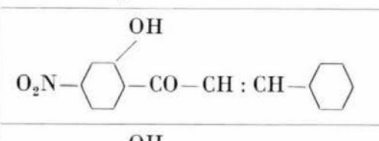
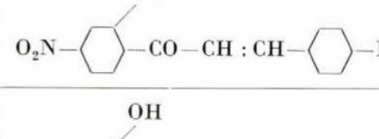
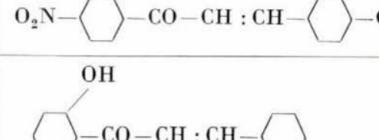
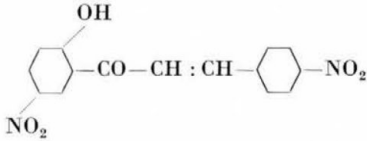
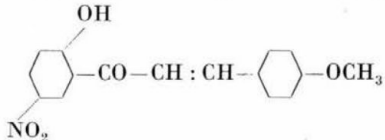
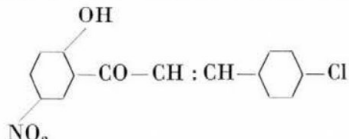
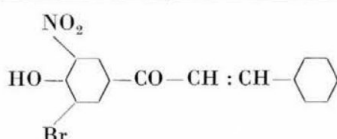
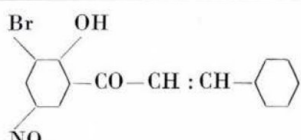
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71.		250	25	12.5	25.8	22.8	—	•	•	•
72.		250	25	12.5	—	—	—	•	•	•
73.		214	21.4	10.7	14.0	—	—	•	•	•
74.		150	15	7.5	16.0	—	—	•	•	•
75.		150	15	7.5	—	—	—	•	•	•
76.		150	15	7.5	—	—	—	•	•	•

Table II continued

No.	Structural formula	Concentration, $\mu\text{g}/0.1 \text{ ml}$			Diameter of inhibition zone, mm		
					<i>Staph. albus</i>		
77.		100	10	5	18.0	—	—
78.		150	15	7.5	—		—
79.		150	15	7.5	—		—
80.		150	15	7.5	24.0	18.2	16.3
81.		150	18.7	9.3	21.6	19.0	15.0

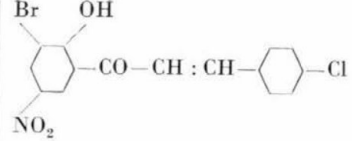
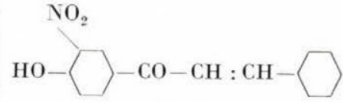
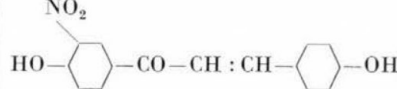
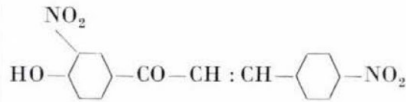
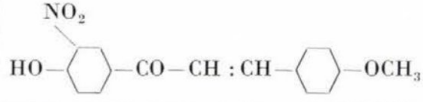
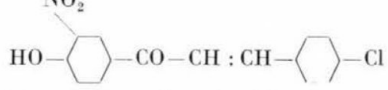
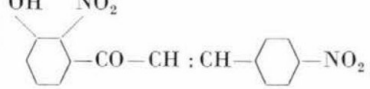
82.		150	15	7.5	19.5	17.8	15.5
83.		166	16.6	8.3	24.0	15.0	—
84.		150	15	7.5	25.0	15.0	—
85.		150	15	7.5	22.5	15.0	—
86.		300	30	15	32.0	30.5	27.6
87.		150	15	7.5	20.5	18.5	15.0
88.		166	16.6	8.3	29.3	20.0	17.0
89.		500	50	25	22.5	15.6	—

Table II continued

No.	Structural formula	Concentration, $\mu\text{g}/0.1 \text{ ml}$			Diameter of inhibition zone, mm		
					<i>Staph. albus</i>		
90.		166	16.6	8.3	—	—	—
91.		150	15	7.5	—	—	—
92.		250	25	12.5	17.0	—	—
93.		150	15	7.5	—	—	—
94.		500	50	25	20.0	18.0	15.0
95.		500	50	25	20.5	17.0	15.0
96.		136	13.6	6.81	—	—	—
97.		166	16.6	8.3	16.6	—	—

Explanations. cr.p. = formation of crystalline precipitate in the agar; — = No effect; * = no clear-cut zone borders;

** = vague but evaluable zone borders; · not tested

The following aldehydes were used for synthesis: 2-, 3-, 4-haloido (Cl, Br, F); 2,4-dichloro-; 4-CN-; 4-NO₂-; 2-methoxy-; 3,4-dimethoxy-; 3,4-diethoxy-; 4-methoxy-; 4-isopropyl-; 4-hydroxy-benzaldehyde; and furfural, piperonal, naphthaldehyde and tetraldehyde. Comparison of the activities of the chalcone derivatives has shown that the ketone part of the derivatives of the four ketones in Fig. 1 displays the following sequence of activity, IV > III > I $\approx$ II.

This would mean that the most favourable structure comprises a hydroxy group in position 4'; those in positions 3' and 2' are intermediately and moderately active, respectively. The position of the nitro group is less important than that of the hydroxyl group, *viz.* chalcones derived from ketones I and II exhibit identical activities, though having the nitro group in different positions. Activities of chalcones derived from ketones II and IV exhibited considerable differences, though both have their nitro groups in identical positions; their hydroxyl groups are, however, in different positions. On the basis of this finding, the similar activity of ketones III and V was not surprising. The practically unimportant effect of the introduction of an NO₂ group into the ketone part is evident from the fact that the activity of —OH chalcones is hardly poorer than that of the corresponding —NO₂—OH chalcones. Our observation concerning the importance of the hydroxyl group's localization was in contrast to that by SCHRAUFSTÄTTER and DEUTSCH [5]. According to these authors, 2'-hydroxy derivatives are more active than 4'-hydroxy ones. The moderate importance of the nitro groups is clear also from their work.

In accordance with SCHRAUFSTÄTTER and DEUTSCH, we found higher activities with —NO₂—OH chalcones derived from Br-substituted ketones, as compared to those not containing Br.

As to the importance of the different substituents of the aldehyde part, the picture is not clear. Nevertheless, a favourable effect of substitution by halogens was observed both by SCHRAUFSTÄTTER and DEUTSCH [5] and by us. Substitution in the ortho-position was regularly favourable, while in the para-position only occasionally so. Of the halogens, fluorine appeared to be more active than bromine and chlorine. Nevertheless, the chalcone derived from ketone III and 2F-benzaldehyde was less potent than expected while 2,4-dichloro derivatives were as active as expected.

All chalcones derived from combinations with 3-nitro-salicylaldehyde were active. This correlates well with the observation of SCHRAUFSTÄTTER and DEUTSCH [5] in that substitution with 2-hydroxyl enhances the bacteriostatic effect.

The action of OH or NO₂ groups at the 4 position of the aldehyde was usually unimportant. The single chalcone analogon obtained from furfural was lacking activity.

Certain chalcones synthesized from alpha naphthaldehyde, p-isopropyl-benzaldehyde or piperonal, were active. The activity of ketone IV was increased

by the presence of a $-\text{OCH}_3$ group in the position 4. No similar effect was observed with other ketones. Chalcone prepared from ketone IV and 2-methoxy benzaldehyde was quite active. This compound appears to be of a certain interest as 4,2', 4'-trimethoxy-chalcone is known to have considerable diuretic and cholagogic effects (cf. "Vesidryl", Laboratories Byla).

LITERATURE

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POST-THERAPEUTIC ANTIBIOTIC CONTENT OF FAECES

By

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(Received October 25, 1966)

Summary. The antibiotic content of faeces of patients receiving chloramphenicol, oxytetracycline and neomycin orally and parenterally has been studied. None of these antibiotics were shown in the faecal samples 24 hours after the discontinuance of parenteral treatment. Similarly, no detectable amounts of orally given chloramphenicol were excreted after 24 hours. After finishing oral administration of oxytetracycline and neomycin, these antibiotics had disappeared from the faeces by the 5th and 7th day, respectively. Half of the patients excreted oxytetracycline on the 4th, and neomycin on the 5th day still in therapeutic concentrations.

Preventive measures for communicable bacterial enteric diseases prescribe that control examinations for the presence of the causative agent should be commenced only after clinical recovery and finishing of antibiotic therapy. There are no regulations as to the exact interval between the discontinuance of treatment and the first bacteriological control examination. This problem may thus give rise to theoretical debates without a sufficient experimental basis. It seemed to us desirable, therefore, to investigate the antibiotic content of faeces after the administration of chloramphenicol, oxytetracycline and neomycin.

Chloramphenicol, owing to its stability and small molecular weight, is readily absorbed from the upper part of the intestinal tract. As soon as 30 minutes after oral ingestion it can be detected in the blood. It disappears from the body within 24 hours. Chloramphenicol is excreted mainly in the urine; therapeutic bile concentrations are, however, reached also easily [1–3]. Following oral administration, the antibiotic can also be detected in the faeces. About one per cent of the total oral dose is excreted in the faeces [4].

Orally given tetracyclines are somewhat less readily absorbed than chloramphenicol. Unlike chloramphenicol, tetracycline in increased doses cause no parallel increase of the serum level; the excess of the antibiotic remains unabsorbed and is excreted in the faeces [3, 5]. Oral tetracycline is excreted in the urine in 15 to 25 per cent [6], according to others in 20 to 55 per cent [7]. The highest amount of the drug is excreted in the faeces. After giving 2 g doses daily, the faecal tetracycline concentration was 2.2 mg/g [8]. After a single dose of 2 g oxytetracycline, the antibiotic was present in the faeces at 1000 µg/g [9]. As chlortetracycline is less rapidly excreted by the kidneys, it appears in the faeces at higher concentrations than other tetracyclines. On the last day

of a therapy administered for several days, the faecal chlortetracycline level as shown by the extraction method, was 4.5 to 21 mg/g [10].

Neomycin is slowly absorbed from the intestinal tract; at most 2 to 3 per cent of the ingested amount enter the circulation [4, 11]. The rest is excreted in a chemically unchanged form in the faeces, where high concentrations may be reached [5, 12].

No data were available as to the duration of the excretion of these antibiotics after the discontinuance of therapy. METZGER *et al.* [10] without presenting quantitative data, reported that the level of aureomycin rapidly decreased when its administration had been discontinued.

Materials and methods

As the purpose of the examinations was to estimate the antibiotics at low faecal concentrations, the tests were performed with low dilutions of the samples. The diffusion method, which in preliminary experiments had proved superior to the tube dilution technique was chosen.

In order to destroy bacteria, the faecal samples were heated to 60 °C for 30 minutes then cooled rapidly. Some surviving organisms, mainly anaerobic bacteria, did not influence the result of the assay. Model experiments showed that the applied heat treatment caused no appreciable decrease in the activity of the antibiotics. After heating, a weighed amount of the sample was suspended in saline.

The test strain was seeded on the surface of agar plates in which then wells 6 mm in diameter were bored. In each well a 0.1 ml aliquot of the appropriate serial dilution of the faecal suspension was pipetted. Every sample was assayed in two parallel experiments. Plates inoculated with *Sarcina lutea* (for chloramphenicol) and with *Staphylococcus aureus* (for oxytetracycline and neomycin) were incubated overnight at 22 °C and 37 °C, respectively. The antibiotic content of the samples was determined by use of a dose response curve obtained by plotting the standard antibiotic concentrations on the logarithmic and the diameters of the inhibition zones on the arithmetic scale of a semilogarithmic paper.

Patients suffering from various diseases were examined. They received chloramphenicol in total doses ranging from 3 to 40 g. Oxytetracycline and neomycin were administered to all patients in a dose of 4 g on one day.

When the drug was given for prolonged periods, the antibiotic content of the faeces was determined once or twice during treatment. After antibiotic had been brought to an end, the faeces were assayed on the 1st, 2nd, 3rd, 4th and 5th day. Some patients, mainly those treated with neomycin, were examined also on further occasions.

The samples were stored in a refrigerator at 4 °C for 2 to 3 hours at most. Most samples were tested within 1 hour.

Results

Oral administration. Excretion of chloramphenicol given orally was studied in 20 patients. None of them were shown to excrete the antibiotic even on the first day after therapy, although some had received a total dose of 40 g of chloramphenicol.

Results obtained in 14 patients treated orally with oxytetracycline are presented in Table I.

Each patient received 4 g oxytetracycline. As shown in Table I, the antibiotic was detected in the faeces of all patients. On the 4th day after therapy

Table I
*Oxytetracycline content of
 faeces after oral administration*

Patient	Oxytetracycline, $\mu\text{g/g}$ faeces; days after discontinuance of therapy				
	1	2	3	4	5
O. I.	100	50	0	0	0
L. I.	350	300	300	200	0
B. F.	300	150	0	0	0
C. M.	350	350	350	150	0
K. M.	200	20	0	0	0
H. J.	20	20	20	0	0
R. J.	300	300	100	50	0
J. I.	20	20	20	10	0
G. J.	200	100	50	10	0
B. B.	150	60	20	0	0
S. P.	100	50	0	0	0
H. J.	280	100	50	10	0
R. G.	20	20	20	20	0
K. A.	250	250	200	0	0

half the samples still contained oxytetracycline. By the 5th day the antibiotic had disappeared from all patients.

Table II shows the results obtained in 20 patients each of which had received 4 g neomycin within 1 day. In the case of 5 patients the post-therapeutic examination was extended to 7 days.

Eleven out of the 20 patients excreted neomycin on the 5th day after discontinuance of therapy. The faeces of 5 patients were examined also on the 6th and 7th day; 2 of them still excreted the antibiotic on the 6th day. By the 7th day none of the patients had detectable amounts of neomycin in their faeces.

Parenteral administration. Five patients received chloramphenicol intramuscularly. The antibiotic was not present in their faeces even on the 1st day after treatment.

None of the 5 patients given oxytetracycline intramuscularly excreted the antibiotic after discontinuance of therapy.

Similarly to the above antibiotics, neomycin was not present in the faeces of 5 patients which had received neomycin parenterally.

Table II
Neomycin content of faeces after oral administration

Patient	Neomycin, $\mu\text{g/g}$ faeces; days after discontinuance of therapy						
	1	2	3	4	5	6	7
V. F.	600	400	400	350	200	.	.
O. J.	600	100	0	0	0	.	.
M. G.	500	400	400	200	0	.	.
C. B.	600	500	50	0	0	.	.
J. K.	600	500	50	0	0	.	.
B. L.	400	0	0	0	0	.	.
K. B.	500	500	500	400	50	.	.
P. J.	500	500	400	0	0	.	.
P. B.	400	400	50	20	20	.	.
B. B.	400	100	20	0	0	.	.
M. L.	400	400	50	50	50	.	.
L. S.	200	50	50	50	20	.	.
O. L.	400	400	300	250	100	.	.
P. K.	400	50	50	50	0	.	.
S. I.	400	400	100	50	0	.	.
B. A.	400	300	150	100	20	0	0
H. V.	400	400	100	50	20	0	0
L. B.	500	400	250	150	100	20	0
S. I.	500	400	300	100	50	40	0
T. A.	400	200	200	100	40	0	0

Discussion

Chloramphenicol, oxytetracycline and neomycin disappeared from the faeces of patients 24 hours after the discontinuance of parenteral administration. Although these studies were carried out in a small number of patients, in view of the identical results we feel justified in drawing conclusions.

In the course of oral administration a considerable difference was observed in the excretion of chloramphenicol and the other two drugs. Chloramphenicol disappeared from the faeces within 24 hours. In contrast, oxytetracycline and neomycin were detected for several days after therapy.

Fig. 1 shows the average of oxytetracycline and neomycin concentration for all faecal samples examined. It is seen that on the 4th day after discontinuance of therapy still $32.1 \mu\text{g/g}$ of oxytetracycline was present in the samples. By the 5th day the antibiotic had disappeared from all patients. Patients treated with neomycin still excreted $33.5 \mu\text{g/g}$ drug on the 5th day. The anti-

biotic had disappeared from all patients by the 7th day. It should be noted that each patient in both groups received 4 g of the corresponding antibiotic on the day preceding the examinations.

There was a direct association between the absorption rate of the drug and the duration of its excretion in the faeces; in other words, the slower the antibiotic is absorbed, the longer it persists in the intestinal tract.

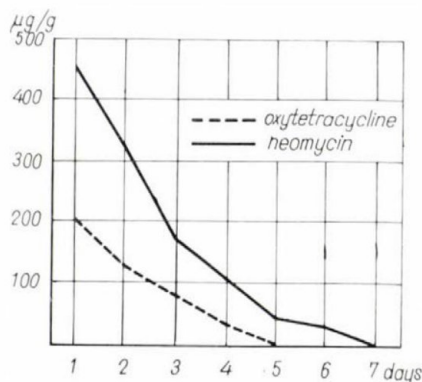


Fig. 1. Oxytetracycline and neomycin concentration in faeces after the discontinuance of therapy. Ordinate: antibiotic concentration in $\mu\text{g/g}$ wet faeces; abscissa: days after therapy. Average data for all samples examined

Although in view of the small number of the examined patients no final conclusions can be drawn, the following considerations seem worth mentioning.

The finding that 4 g neomycin given within 24 hours was present at therapeutic concentrations in the faeces for as long as 5 days means that in reality the patient received a prolonged antibiotic treatment lasting for several days. This, as well known, may give rise to therapeutic side effects such as an impairment of the normal intestinal flora. On the other hand, the prolonged excretion of the drug may cause diagnostic errors in bacteriological examinations. In the light of our findings, the results of faecal cultures seems unreliable if the two compulsory control examinations of patients recovered from dysentery are performed as early as on the 2nd and 4th or even on the 3rd and 5th days after discontinuance of therapy. The same consideration is more or less valid for oxytetracycline.

Acknowledgement. The authors wish to thank Mrs. F. PARRAGH for skilled technical assistance.

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DEFEKTIV LYSOGENE BACILLUS ANTHRACIS-STÄMME

Von

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(Eingegangen am 26 Oktober, 1966)

Zusammenfassung. Aus der in CO₂-Atmosphäre bereiteten Kultur eines mit einer virulenten Mutante des W-Phagen infizierten kapsulogenen *B. anthracis*-Stammes konnten defektiv lysogene Isolate gewonnen werden. Diese defektive Prophagen tragenden akapsulogenen Mutanten setzen Phagen nicht frei, und nur ihre Immunität W-Phagen gegenüber läßt auf ihre lysogene Natur schließen. Entgegen diesen defektiv lysogenen Stämmen adsorbiert das phagenresistente Isolat die homologen Phagen nicht. Aus den W-Phagen konnten temperierte und virulente Mutanten isoliert werden, die mit den defektiven Stämmen einen lysogenen Komplex bilden oder diese Zellen auflösen.

MCCLOY [1] hatte 1951 aus dem einen lysogenen Stamm des *Bacillus cereus* einen temperierten Phagen isoliert, der verschiedene *Bacillus anthracis*-Stämme sehr spezifisch beeinflußte. Er nannte diesen Phagen und seine Mutanten W-Phagen. Mit diesem Phagen befassen sich zahlreiche Mitteilungen [2, 3, 4, 5, 6, 7,]. Den aus dem lysogenen *B. cereus*-Stamm freiwerdenden, temperierten Phagen bezeichnete MCCLOY mit β , und seine Mutanten mit α und γ . Mit dem β -Phagen sind der atypische, asporogene Davis-Stamm des *B. anthracis* und die typischen Anthraxstämme gleichermaßen lysogenisierbar. MCCLOY vermochte mit dem α -Phagen lysogene Bakterien nicht zu erhalten [2]. Unlängst hatte ich mitgeteilt, daß mitunter auch mit der α -Mutante mit typischen Anthraxstämmen lysogene Komplexe hergestellt werden konnten. Die mit dem Vollum-Stamm des *B. anthracis* gewonnenen α -lysogenen Komplexe haben sich als äußerst labil erwiesen, und der Prophag wird durch Kohlendioxyd induziert. Eine weitere Mutante des α -Phagen ist αC , mit der Bakterien nicht zu lysogenisieren waren [8].

Ich beobachtete, daß mit dem αC -Phagen unter entsprechenden Bedingungen Isolate zu erhalten sind, welche nicht nur gegen die α - und β -, sondern auch gegen die αC -Phagen immun sind. Obwohl die W-Phagen diese Isolate nicht angreifen, werden die Phagen doch ausgiebig adsorbiert, sie sind also nicht als resistent, sondern als immun zu betrachten. Diese immunen Stämme lassen aus ihrem Inneren keine infektiösen Phagenteilchen freiwerden, so daß für die Immunität lediglich defektive Prophagen verantwortlich sein können.

Die vorliegende Mitteilung schildert die Isolierung der defektiv lysogenen *B. anthracis*-Stämme und einige ihrer Eigenschaften.

Material und Methoden

Zu den Versuchen wurden kapsulogene VC^+ - und akapsulogene VC^- -Varianten des Vollum-Stammes sowie der Davis-Stamm benutzt. Im Laufe früherer Arbeiten [7, 8] hatten wir das Derivat $VC^-/128$ isoliert, welches sich als eine dem W-Phagen gegenüber resistente Mutante des Vollum-Stammes erwies.

Über die benutzten W-Phagen gibt die vorangegangene Arbeit [8] Aufschluß. Die Phagen A_2 und A_3 sowie ihre Eigenschaften haben wir unlängst beschrieben [9], und die Mitglieder dieser beiden Phagengruppen unterschieden sich scharf von den W-Phagen.

Eine Beschreibung der Nährböden und der angewandten Methoden enthalten frühere Mitteilungen [7, 8].

Ergebnisse

Isolierung defektiv lysogener Stämme. Von dem VC^+ -Sporenmaterial wurden auf 50% Pferdeserum enthaltendem YP-Agar diagonale Abimpfung vorgenommen und darüber kreuzweise mehrere parallele Linien mit einer αC -Phagenverdünnung von 10^{-4} gezogen. Die Kulturen wurden 96 Stunden in Thermostat in 25%iger CO_2 -Atmosphäre inkubiert. Aus den stark mukoiden Kolonien gingen trockene Fortsätze hervor. Nur wurde an verschiedenen Stellen Sporenmaterial entnommen und das Probegemisch nach 5 Minuten Erwärmen bei $80^\circ C$ in Davis-Indikator und auf Agarplatten ausgebreitet. Auf den mit Davis-Indikator bereiteten Platten kamen lysogene Klone nicht zur Beobachtung. Das Sporenmaterial lieferte akapsulogene Kolonien (in der CO_2 -Atmosphäre), die sich gegenüber den αC -Phagen teils als resistent, und teils als empfindlich erwiesen. Die meisten der resistenten Kolonien trieben ungewöhnlich stark und schnell Sporen, wodurch die Kolonien porzellanweiße Farbe erhielten. Von diesen Kolonien wurden weitere Ausbreitungen hergestellt und von den in der Subkultur gefundenen Kolonien einige wieder auf ihre Phagenempfindlichkeit geprüft. Die aus den resistenten Kolonien bereiteten zweiten Subkulturen segregierten schon keine phagenempfindlichen Kolonien mehr. Auf solche stabilen Isolate beziehen sich unsere Beobachtungen.

Die Eigenschaften der phagenresistenten Isolate. In zwei gesonderten Versuchen wurden 14 resistente Isolate untersucht. Bei der Ausbreitung des Sporenmaterials dieser Isolate auf $10^4/ml$ αC -Phagenteilchen enthaltende Agarplatten wurde die gleiche Zahl von Kolonien gefunden wie im Falle der phagenfreien Kontrollplatten. Somit schienen die Isolate homogen zu sein.

Die Morphologie der Kolonien der phagenunempfindlichen Isolate unterschied sich von der des parenten Stammes, sie waren etwas kleiner und unregelmäßiger als die VC^- -Kolonien. In zahlreichen Versuchen trachteten wir unter verschiedenen Bedingungen Phagen aus den Isolaten nachzuweisen. Im Falle der mit dem Davis-Stamm hergestellten gemischten Kulturen gelang dies auch dann nicht, wenn vorher die resistenten Stämme verschieden lange Zeit mit UV-Licht bestrahlt oder mit Mitomycin-C behandelt worden waren. Trotz ihrer Resistenz gegen W-Phagen haben sie diese Phagen ausgiebig adsor-

biert, während von dem früher isolierten resistenten Derivat $VC^-/128$ W-Phagen überhaupt nicht gebunden wurden. Der allen W-Phagen gegenüber resistente Stamm erwies sich den heterologen A_2 - und A_3 -Phagen gegenüber als empfindlich. Dies demonstrieren die »e. o. p.« (efficiency of plating)-Werte (s. Tabelle I).

Tabelle I

Der e. o. p.-Wert des homogenen αC - und der heterologen A_2 - und A_3 -Phagen im Falle des nicht lysogenen, des defektiv lysogenen (dl) und des resistenten ($VC^-/128$) Stammes, sowie die Adsorption des αC -Phagen

Stamm	E. o. p.-Werte mit verschiedenen Phagen			Adsorption des αC -Phagen %
	αC	A_2	A_3	
Davis	1,0	1,0	1,0	96
VC^-	0,8	0,7	1,0	95
5 VC^-dl	10^{-5}	1,0	1,0	92
6a VC^-dl	10^{-5}	1,0	1,0	92
6b VC^-dl	10^{-5}	1,0	1,0	90
7 VC^-dl	10^{-6}	1,0	1,0	74
8 VC^-dl	10^{-5}	1,0	1,0	88
9 VC^-dl	10^{-7}	1,0	1,0	82
10 VC^-dl	10^{-6}	1,0	1,0	87
11 VC^-dl	10^{-6}	1,0	1,0	85
$VC^-/128$	$<10^{-7}$	1,0	1,0	0,0

Auf Grund der verschiedenen Evidenzen ist der $VC^-/128$ -Stamm als »echter resistenter« Stamm zu betrachten, während die übrigen immun für den homologen Phagen sind. Da es nicht gelang, aus unseren Stämmen infektiösfähige Phagen zu isolieren, sind die Stämme als defektive Lysogene zu werten. Deshalb nehmen wir im weiteren und in Tabelle I hierauf mit der Bezeichnung *dl* Bezug. Die Ziffern bedeuten die einzelnen Isolate.

Ein Teil der *dl*-Stämme lieferte für das αC -Phagenmaterial Plaques mit sehr niedrigen e. o. p.-Werten. Durch Propagieren der aus den Plaques erhaltenen Phagen auf seinem eigenen defektiv-lysogenen Stamm oder auf dem Davis-Stamm ließ uns zu einem Phagenmaterial gelangen, dessen e. o. p.-Wert dem der nicht lysogenen und *dl*-Stämme analog war. Das heißt, hier hatten wir Phagenmutanten vor uns, die auf nicht lysogenen und defekt-lysogenen Stämmen gleichermaßen gut gediehen. Gleichzeitig blieb der $VC^-/128$ -Stamm auch für diese Phagen resistent. Demnach verleiht der defektive Prophage dieser Mutante gegenüber, die wir im weiteren h-Mutante nennen, keine Immunität.

Auch die β -Mutanten des W-Phagen bildeten auf den *dl*-Isolaten Plaques mit sehr niedrigen e. o. p.-Werten. Einen aus dem Stamm 5VC⁻ *dl* gewonnenen Phagen haben wir aus einem Plaque in Davis-Stamm propagiert und sein Wirkungsspektrum auf Grund der e. o. p.-Werte kontrolliert.

Tabelle II

*E. o. p.-Werte des β -Phagen und seiner auf dem Stamm 5VC⁻ *dl* erhaltenen Mutante (βh) bei den Stämmen VC⁻ und 5VC⁻ *dl**

Phag	E. o. p.-Werte bei den Stämmen	
	VC ⁻	5VC ⁻ <i>dl</i>
β	1,0	10^{-5}
βh	1,0	1,0

Wie ersichtlich, konnte auch aus β -Phagen eine *h*-Mutante isoliert werden, welche sich für den ursprünglichen Stamm und die *dl*-defektiv lysogenen Stämme als gleichermaßen wirksam erwies.

Mit dem βh -Phagenmaterial konnten sowohl der originale VC⁻-Stamm als auch die *dl*-Stämme lysogenisiert werden. Der mit βh -Prophagen aus dem lysogenen VC⁻ *dl* (βh)-Stamm freigewordene Phage stimmte hinsichtlich seiner Antigenstruktur mit dem β - und dem α C-Phagen überein.

Besprechung

Auf Grund der Studien von BUCK und Mitarbeitern [10] wissen wir, daß sich unter den in der Natur vorkommenden *B. anthracis*-Stämmen auch lysogene befinden können. Aus 25 der zahlreichen Laboratorien entstammenden 139 Sammelstämmen vermochten sie Phagen zu isolieren. Mit diesen Phagen sowie mit dem γ -Phagen unternahmen sie den Versuch einer Typisierung von 246 verschiedenen *B. anthracis*-Stämmen. Ein Teil derselben erwies sich als resistent gegenüber allen oder einzelnen Test-Phagen. Die Bemühungen, aus einigen solchen resistenten Stämmen Phagen zu isolieren, blieben jedoch erfolglos.

Unsere Beobachtungen deuten darauf hin, daß im Falle des *B. anthracis* nicht nur mit typischen lysogenen Stämmen, sondern auch mit solchen gerechnet werden muß, die Träger defektiver Prophagen sind. Gegenüber seinen homologen Phagen sichert auch der defektive Prophag eine Immunität [11]. Zahlreiche Mitteilungen [1, 5, 10, 12, 13] berichten darüber, daß sich unter den *B. anthracis*-Stämmen auch verschiedenen Phagen gegenüber resistente befin-

den können. Wie wir sahen, ist im Falle einer Resistenz gegen die Phagen mit zwei Mechanismen zu rechnen: (1) mit dem Fehlen des Rezeptors, welches ein Analogon zu der beim T-Phagen-System erkannten Resistenz ist, und (2) mit der seitens des Prophagen gebotenen Immunität. Der Umstand, daß die *B. anthracis*-Stämme auch defektive Prophagen tragen können und daher in ihren Kulturen infektiöse Teilchen nicht nachweisbar sind, macht es nötig, daß zwecks Ermittlung der beiden möglichen Mechanismen mit den resistenten Stämmen auch Untersuchungen bezüglich der Phagenadsorption angestellt werden.

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FATTY ACID COMPOSITION OF STAPHYLOCOCCUS AUREUS STRAINS DIFFERING IN ANTIBIOTIC SENSITIVITY

By

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(Received November 11, 1966)

Summary. The total fatty acid content and fatty acid spectrum of *Staphylococcus aureus* strains with different antibiotic sensitivity have been examined.

Antibiotic sensitive and resistant strains did not differ significantly in total fatty acid content. The examined cultures synthesized the same fatty acids. The main components were anteiso-C₁₅, arachic acid, C₁₇ cyclopropane-ring containing or branched chain fatty acid and stearic acid.

Antibiotic sensitive and resistant staphylococci, which had been cultured for 18 hours under strictly identical conditions, differed in the percentage distribution of fatty acids. In sensitive strains C₁₇ or C₁₉ cyclopropane-ring containing or branched chain fatty acids comprised 16 to 20 per cent of the total fatty acid content. The same substances occurred in resistant strains in 33 to 44 per cent.

The lipid metabolism and lipid composition of *Staphylococcus aureus* have been scarcely investigated and the data available are often contradictory. POLONOVSKY *et al.* [1] found the lipids of *Staph. aureus* to consist of nitrogen-free lipids and glycolipids. MAC FARLANE [2] and HOUTSMULLER and VAN DEENEN [3] demonstrated that *Staph. aureus* synthesized nitrogen-containing complex lipids and the main part of its phosphatides was composed of phosphatidyl glycerol and its *o*-lysine ester. HOUTSMULLER and VAN DEENEN [4] showed that the proportion of these components varied with culturing conditions; in acid medium the phosphatidyl glycerol lysine ester content increased and the amount of phosphatidyl glycerol decreased. VÁCZI *et al.* [5] demonstrated that antibiotic resistant *Staph. aureus* strains contained more total lipid and phosphatide than sensitive strains. As to the fatty acid composition of *Staph. aureus* only one paper, MAC FARLANE's short communication [6], was available. The purpose of the present study was to reveal differences in the fatty acid composition of strains differing in antibiotic sensitivity and to determine fatty acids synthesized by staphylococci obtained from various sources.

Materials and methods

Organisms. *Staphylococcus aureus* strains examined in this study uniformly produced coagulase and phosphatase, split mannitol, liquefied gelatin and were haemolytic. The following strains were used. Antibiotic sensitive type strain SG 511 and strain 100 of our collection;

polyresistant strain 53 of our collection and its mutants*; methicillin resistant strains 18 R, SP-26367 and B-24373**.

Culturing technique. Strains differing in antibiotic sensitivity were cultured under identical conditions in the following medium. Tripkazin (pancreatic digest of casein, Human, Budapest), 10 g; NaCl, 5 g; Na_2HPO_4 , 5 g; ferrous ammonium sulphate, 0.03 g; MgSO_4 , 0.01 g; MnSO_4 , 0.01 g; glucose, 5 g; vitamin B₁, 10 μg ; nicotinamide, 10 μg ; distilled water to 1000 ml. Glucose, vitamin B₁ and nicotinamide were sterilized by filtration and added to the rest of the medium before seeding. The medium was adjusted to pH 7.2.

The cultures were shaken for 18 hours at 37 °C in 500 ml Erlenmeyer flasks containing 150 ml medium each. The bacteria were then centrifuged at room temperature for 20 minutes at 3000 r. p. m. and washed in physiological saline. Extraction of fatty acids and gas chromatography were performed as described previously [7].

Results

Antibiotic sensitivity of the strains and their total fatty acid content expressed in weight per cent of dry cells are shown in Table I.

Table I
Antibiotic sensitivity and total fatty acid content of Staph. aureus strains

Strain	Antibiotic sensitivity						Fatty acid content*
	P	S	C	T	E	M	
SG 511	S	S	S	S	S	S	3.2
100	S	S	S	S	S	S	3.0
53	R	R	R	R	R	S	2.5
53/1	R	R	S	R	R	S	2.3
53/5	R	R	S	R	R	S	2.3
53/101	R	R	S	S	R	S	2.2
53/300	R	R	S	S	R	S	2.3
18 R	R	R	R	R	R	R 100 $\mu\text{g}/\text{ml}$	3.0
SP-26367	.	.	.	.	.	R 40 "	3.4
B-24373	.	.	.	.	.	R 60 "	3.4
80/81	R	R	R	R	R	S	3.3

* Expressed as weight per cent of dry bacteria: average result of 3 parallel experiments. Abbreviations: P = penicillin, S = streptomycin, C = chloramphenicol, T = tetracycline, E = erythromycin, M = methicillin, S = sensitive, R = resistant.

On the basis of their antibiotic sensitivity, the organisms could be divided into 4 groups, viz. (1) antibiotic sensitive strains; (2) polyresistant, methicillin sensitive strains; (3) mutants of the polyresistant strain with different antibiotic sensitivity; (4) polyresistant and methicillin resistant cultures.

Resistance to methicillin expressed in Table I as the minimal inhibitory concentration, was determined by the tube dilution technique. From data show-

* Mutants of strain 53 showing different antibiotic sensitivity were selected and kindly supplied by Dr. M. FODOR of our institute.

** Strains 18 R and SP-26367 were kindly supplied by Dr. M. BARBER, strain B-24373 by Dr. G. T. STEWART (England).

ing total fatty acid contents it is evident that in this respect there was no significant difference between our strains. Lower values were obtained for strain 53 and its mutants.

The strains were similar in fatty acid spectrum; they differed, however, in the proportion of various components.

Gas chromatographic analysis revealed that *Staph. aureus* contained 13 different fatty acids. The percentage distribution of fatty acids in antibiotic sensitive strains is presented in Fig. 1.

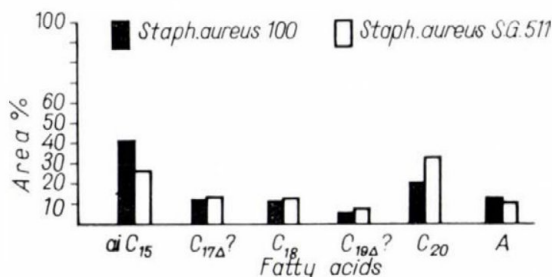


Fig. 1. Fatty acids in *Staph. aureus* strains 100 and SG 511. Percentage of areas recorded by the W. G. Pye Argon chromatograph without correction factors. A = Total of C_{14:1}; C₁₄; C_{16:1}; C₁₆; C_{18:1}; C₁₉; unknown I; unknown II

It is seen that in antibiotic sensitive strains 26 to 42 per cent of the total fatty acid content was constituted of anteiso-C₁₅ fatty acid. The second main component, arachic acid (C₂₀), was present in 25 to 35 per cent. Stearic acid was found in 14 to 15 per cent. In sensitive strains two, not fully identified, components were demonstrated: C₁₇ and C₁₉ branched-chain or cyclopropane-ring containing fatty acids. Further components, namely C_{11:1}, miristic, palmitoleic, palmitic, oleic and linoleic acids comprised 10 to 15 per cent of the total fatty acid content.

Fig. 2 indicates the percentage distribution of fatty acids in *Staph. aureus* strain 53 and its mutants. In fatty acid spectrum these cultures were similar to sensitive strains. There was a difference in the proportion of the various components. Anteiso-C₁₅ fatty acid was present in 45 to 55, the non-identified C₁₇ cyclopropane-ring containing or branched-chain fatty acid in 28 to 30, and arachic acid in 4 to 5 per cent.

Fig. 3 shows the distribution of fatty acids in staphylococci with different methicillin sensitivity. Strain 18 R, which exhibited the highest resistance to methicillin, contained cyclopropane-ring containing or branched-chain fatty acids in 44 per cent. These special fatty acids were shown in strains SP-26367 and B-24373 in 32 and 35 per cent, respectively.

A more precise identification of special fatty acids, especially of the 17 carbon atom component, seemed desirable. The indirect method was chosen

for demonstration of the cyclopropane structure. The C_{17} fatty acid of *E. coli* 0111, which had been shown to be a cyclopropane-ring containing compound [8, 9], was isolated by gas chromatography. This substance was, as a known component, added to a fatty acid preparation obtained from *Staph. aureus*.

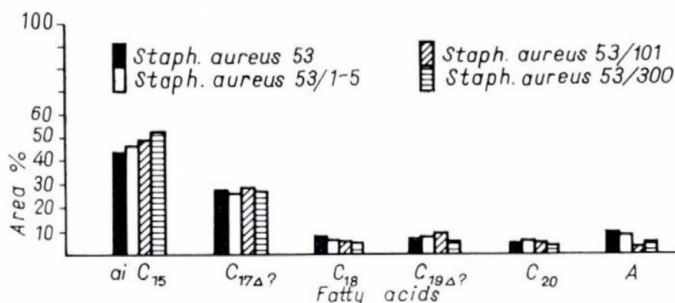


Fig. 2. Fatty acids in *Staph. aureus* strain 53 and its mutants. Percentage of areas recorded by the W.G. Pye Argon chromatograph without correction factors. A = Total of $C_{14:1}$; C_{14} ; $C_{16:1}$; C_{16} ; $C_{18:1+2}$; $C_{19?}$; unknown I; unknown II

Chromatograms presented in Fig. 4 indicate that after the addition of *E. coli* C_{17} cyclopropane fatty acid to staphylococcal fatty acids there was an increase in the peak regarded as characteristic of C_{17} cyclopropane fatty acids (part B of Fig. 4.). It is therefore probable that *Staph. aureus*, too, synthesizes cyclopropane fatty acid(s). A final conclusion shall be drawn from our infrared spectroscopic examinations which are in progress. Grouping of staphylococcal

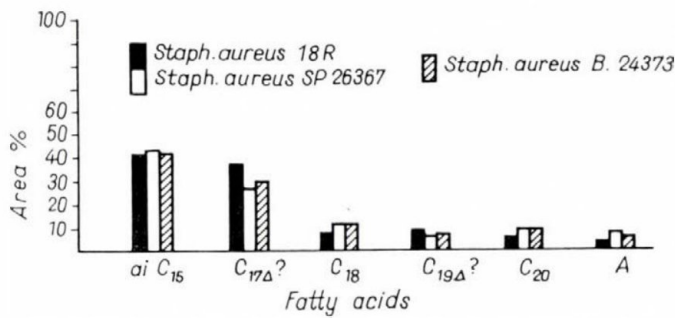


Fig. 3. Fatty acids in methicillin resistant *Staph. aureus* strains. Percentage of areas recorded by the W. G. Pye Argon chromatograph without correction factors. A = Total of $C_{14:1}$; C_{14} ; $C_{16:1}$; C_{16} ; $C_{18:1+2}$; $C_{19?}$; unknown I; unknown II

fatty acids according to structure revealed the following differences between the strains.

From Fig. 5 it is evident that with the broadening of antibiotic resistance pattern there is an increase in special cyclopropane-ring containing or branched-chain fatty acids and a decrease in unsaturated and saturated straight-chain fatty acids.

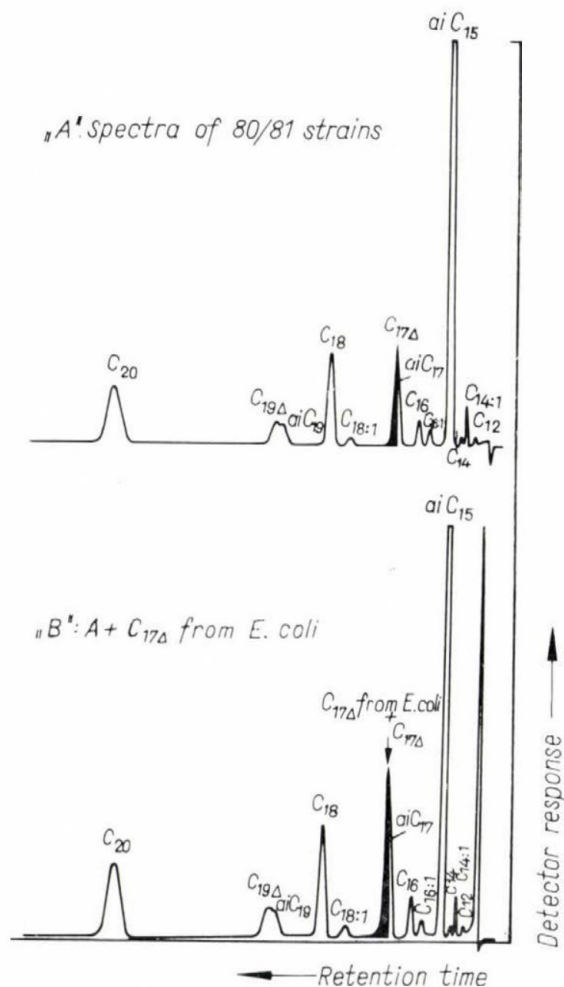


Fig. 4. Fatty acid spectra of *Staph. aureus* 80/81 strains. W. G. Pye Argon chromatograph; beta ray ionization detector; Celit 545 (100—120 mesh) column supplied with 10 per cent APL; temperature: 200 °C; flow rate; 60 ml/minute; detector voltage: 1250 V

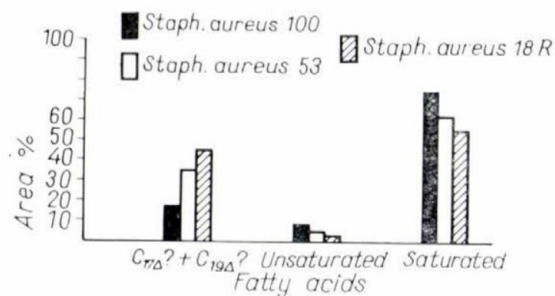


Fig. 5. Comparison of main fatty acids in *Staph. aureus* strains with different antibiotic sensitivity. Percentage of areas recorded by the W. G. Pye Argon chromatograph without correction factors

Discussion

According to MAC FARLANE [6] the lipids of *Staph. aureus* contain the following fatty acids: anteiso- C_{15} (45 per cent), iso- C_{15} (13 per cent), anteiso and iso- C_{17} (12 per cent) and normal saturated fatty acids C_{16} (9 per cent), C_{18} (9 per cent) and C_{20} (3 per cent).

Our staphylococcal strains were similar to those of MAC FARLANE in containing 24 to 54 per cent anteiso- C_{15} fatty acid. Further main components in our antibiotic sensitive strains which together with anteiso- C_{15} comprised 90 per cent of the total fatty acid content, were arachic (20 to 32 per cent), $C_{17\Delta?}$ (12 to 13 per cent) and stearic acid (11 to 12 per cent). Antibiotic resistant strains contained these substances in different proportions. Special (cyclopropane-ring containing or branched-chain) compounds occurred in these strains in 34 to 44 per cent and constituted therefore the second main fatty acid component. Arachic acid was present in 3 to 5 per cent.

The difference between MAC FARLANE's and our findings may probably be attributed to differences in culturing technique and staphylococcal strains.

The quantitative composition of bacterial fatty acids is influenced by the ingredients and pH of the culture medium, temperature and duration of incubation, etc. Bacteria cultured at lower temperatures contain higher amounts of unsaturated fatty acids [10]. A change in the ingredients, pH and oxygenization of the medium results in the alteration of cyclopropane-ring containing fatty acids in *E. coli* [11].

The fatty acids of our staphylococcal strains were determined under standard conditions and therefore the quantitative differences observed can be explained by a different lipid metabolism.

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ANNUAL MEETING OF THE HUNGARIAN ASSOCIATION OF MICROBIOLOGISTS

Debrecen, September 5—7, 1966

ABSTRACTS OF PAPERS

BACTERIOLOGY

I. SZABÓ

(*Korányi Institute for Tuberculosis, Budapest*)

DIAGNOSIS, INCIDENCE AND EPIDEMIOLOGICAL IMPORTANCE OF ATYPICAL MYCOBACTERIA

In recent years an increasing importance has been attached to the spread and precise identification of atypical mycobacteria, particularly in the field of veterinary medicine, especially in view of the economic aspects. In human medicine, their frequency has decreased and their investigation has thus been less extensive. Still, the study of their properties and attempts at reliable diagnosis are still justified.

Numerous well known tests have been applied for the diagnosis of atypical mycobacteria. Development and adaptation for routine work of the iron uptake test has been carried out by Hungarian workers. This reaction has been routinely adopted in Europe as well as in the USA. Recently the test has been shown to allow some conclusions as to the facultative pathogenicity of the strains.

All hitherto identified strains have been isolated in this country from various biological specimens, and have been shown to play a causative role in both human and veterinary tuberculosis. From the veterinary point of view it is of particular importance that animals infected with these strains give cross reactions with both human and bovine tuberculin and may be emergency slaughtered for this reason. Accordingly, further investigations of this group of mycobacteria are not only of theoretical but also of practical importance.

J. VÁNDOR, F. GIMPL

(*Department of Pulmonary Diseases, University Medical School, Budapest*)

BACTERIOLOGICAL, BIOCHEMICAL AND IMMUNOCHEMICAL COMPARATIVE STUDIES ON PHOTOCHROMOGENIC MYCOBACTERIUM KANSASII AND ITS SCO- TOCHROMOGENIC VARIANTS

TACQUET, TISON and DEVULDER (Lille) have isolated scotochromogenic variants of *M. kansasii*. Pigment production, photo- and scotochromogenicity being one of the fundamental characters for the classification of atypical

mycobacteria five photochromogenic and three scotochromogenic strains of *M. kansasii* have been examined for bacteriological and biochemical properties. The antigenic structure of the two strains was compared by immune diffusion, immune electrophoresis and immune absorption. The strains were found to be identical except in pigment production. Accordingly, classification of atypical Mycobacteria should be based on the comparison of their antigenic structure and on BOENICKE's amide series.

F. GIMPL, J. VÁNDOR

(Department of Pulmonary Diseases, University Medical School, Budapest)

TYPING OF MYCOBACTERIUM KANSASII BY IMMUNOCHEMICAL METHODS

Using the methods of immune-diffusion, immune-electrophoresis and immune-absorption, the antigenic structure of atypical *M. kansasii* strains was compared to those of *M. tuberculosis*, *M. bovis*, *M. avium* strains belonging to groups Runyon II, III and of some rapidly growing saprophytic mycobacteria. The antigenic structure of *M. kansasii* resembled that of *M. tuberculosis* and *M. bovis*, but differed from that of the others. A specific antigenic component was demonstrated for *M. kansasii* strains following the absorption of anti-kansasii immune serum with *M. tuberculosis* antigen. The components were characterized by immune electrophoresis. The *M. kansasii* strains were typed by demonstrating characteristic antigenic components.

S. TUBOLY

(Central Veterinary Institute, Budapest)

HEAT-RESISTANT ANTIGENIC FACTORS OF PATHOGENIC MYCOBACTERIA

The heat resistant antigenic factors of pathogenic mycobacteria have been studied in order to clarify whether monospecific fractions can be isolated from tuberculoproteins, viz. to identify the antigenic components responsible for the cross reactions observed in immune diagnostics.

By electrophoresis (1500 V/40 mA) on Whatman's No. 1 paper of 8 week Seitz filtrates of mycobacterium strains grown on Sauton agar, the number of antigenic factors and their electrophoretic mobility has been determined. Heat resistant factors were studied in heat denatured filtrates.

From the filtrate of *M. bovis*, 6 fractions were separated at pH 4.2; two migrated towards the negative, four towards the positive pole. Under similar conditions, the protein constituents of *M. avium* were separated into 5 fractions; one remained at the starting point, one migrated towards the negative and 3 towards the positive pole.

The fractions thus obtained were eluted from the paper strip and their protein content was adjusted to 0.5 mg/ml. The immunochemical purity of the fractions was checked by gel precipitation and immune-electrophoresis. The immune-biological activity of the purified fractions was assayed in guinea-pigs infected with *M. bovis*, respectively *M. avium*.

Under these conditions the tuberculoproteins were separable into peptides, some of which gave a positive reaction only in the case of allergy elicited by the homologous strain.

B. RALOVICH, S. VÖRÖS

(Institute of Microbiology, University Medical School, Pécs)

A NEW ANTIGENIC RELATIONSHIP BETWEEN *E. COLI* AND *M. MORGANII*

The biochemical and antigenic characters of a *M. morganii* strain isolated by the authors are reported. A close antigenic relationship was found to exist with the serotypes 0111ab:K58(B4) and 0111ac:K58(B4) of *E. coli*. One of the factors responsible for the "O" antigen relationship is the component "a" present in all the three strains. The other factor is part of component "b", present in *E. coli* 0111ab. The "b" antigen comprises two parts, b₁ and b₂. The *E. coli* 0111ab strain contained both, while the strain *M. morganii* contained only "b₂". Thus the somatic antigen of the three strains may be symbolized as follows. 0111ab = ab₁b₂; 0111ac = ac; *M. morganii* = 0111ab₂.

The structure of the surface antigen is more complicated. The "K" antigen of the three strains may be symbolized as follows. 0111ab:K58(B4) = Ba, Bb, (Bd); 0111ac:K58(B4) = Ba, Bc, (Bd); *M. morganii* = Ba, Bd (where B symbolizes the surface antigen and letters in brackets are incomplete antigens). Though the surface antigen of *M. morganii* is closely related to antigen K58(B4), functionally it differs from the latter, since it enhances agglutinability. The flagellar antigen of the strain differs from those hitherto described.

É. CZIROK, M. HARASZTI, A. PETRILLA, I. PERÉNYI

(National Institute of Public Health, Public Health Station of County Pest, Budapest, and Municipal Hospital, Vác)

E. COLI 018a, 18c:K77(B21):H7 ENTERITIS AMONG NEWBORNS

An outbreak of enteritis associated with mild symptoms has been observed; it lasted for two months and involved a total of 98 babies. Seventy-four faecal samples taken from 40 sick babies and from 14 members of the nursing staff were examined. *E. coli* showing late lactose fermentation was isolated in almost pure culture from 37 faecal specimens of 24 babies. The antigenic structure of the agent was 018a, 18c:K77(B21):H7. In Hungary, no outbreak associated with this strain has so far been reported.

J. MÉSZÁROS, L. STIPKOVITS

(Research Institute of Veterinary Medicine of the Hungarian Academy of Sciences, Budapest)

SPREAD OF *E. COLI* IN POULTRY FLOCKS INFECTED WITH *MYCOPLASMA GALLISEPTICUM*

In poultry flocks infected with *Mycoplasma gallisepticum*, a bird-to-bird spread of *E. coli* infection has been observed. Transmission of *E. coli* by contact was evidenced by temperature elevation, clinical signs, and death, serological positivity and presence of typical gross lesions in the contact birds, as well as by the identification of the *E. coli* strains isolated from the carcasses. Among the chickens infected intratracheally with *E. coli*, 90 per cent had elevated temperatures and 90 per cent a serological immune response. Among the contacts, 40 per cent had temperature elevation and 30 per cent developed a serological response. Fatality rates were approximately 40 and 10 per cent in the intratracheally infected and the contact groups, respectively.

E. KERÉKES, I. RASKÓ, L. ALFÖLDI

(Institute of Microbiology, University Medical School, Szeged)

INDUCTION OF L-SERINE DEAMINASE OF *E. COLI*

In the last year we have reported on our hypothesis that the origin of amino acid sensitivity of certain *E. coli* strains might be an induction or structural anomaly of the L-serine deaminase and L-threonine deaminase enzymes.

Based on this hypothesis optimal conditions for *E. coli* L-serine deaminase induction have been studied. A combination of L-leucine and L-glycine was found to be optimal, while its substrate, L-serine, was inactive in this respect. This peculiar behaviour is difficult to explain on the basis of the available theories and for its clarification further experiments are needed.

B. LÁNYI, M. GREGÁCS, M. ÁDÁM

(National Institute of Public Health, Budapest)

INCIDENCE OF *PSEUDOMONAS AERUGINOSA* SEROGROUPS IN WATER AND HUMAN FAECES

Bacterial examination of 9219 drinking water, 1065 river water and 419 sewage samples, and faecal specimens of 7650 healthy adults and 650 hospitalized infants supplied 521 *Ps. aeruginosa* strains. The percentage frequency of *Ps. aeruginosa* was 1.6 in wells, 4.1 in springs, 0.6 in piped water, 1.8 in river and 10.0 in sewage samples. The faeces of healthy adults living in the same geographic area were positive in 1.5 per cent. Hospitalized infants harboured the organism in 32.0 per cent.

The serogroup distribution of *Ps. aeruginosa* was very similar in water and adults' faecal samples. From both groups of material serogroups 1, 3, 4, 5 and 6 were isolated most frequently. This finding indicates that the presence of *Ps. aeruginosa* in drinking water is probably the result of faecal contamination. A comparison of the data with other studies has shown that, although in a somewhat different distribution, the above serogroups of *Ps. aeruginosa* are most commonly encountered in intestinal and extraintestinal infections. The results support the opinion that waters containing this organism are unsatisfactory for human consumption.

J. SZITA, GY. HEGYESSY

(National Institute of Public Health, Budapest)

TYPE DISTRIBUTION OF STREPTOCOCCUS PYOGENES STRAINS IN 1964-65

In cooperation with the International Subcommittee on Streptococci and Pneumococci, 395 *Streptococcus pyogenes* strains were collected by 11 competent Hungarian institutions over the one-year period 1.6.1964 - 1.6.1965. The strains belonged to 26 different types. Only 0.8 per cent of the strains were untypeable by agglutination and precipitation. The predominant (5 per cent, or more) strains were complexes 5, 11, 12..., 3, 13, B₃₂₆₁, and types 3, 12, 1 and 6. These results are in good correlation with the known literary statement that - except for nephritis - the disease has no relationship with the type of the causative *Streptococcus pyogenes* strain. Out of 19 strains typed by us and submitted for control to the Prague Centre, 4 types were inconsistent and sent therefore to Genua for further identification. All the 4 reacted with the same agglutinating sera as in our laboratory but 3 of them also with anti-serum 28. Consistent results were obtained with the strains submitted to us for control from Genua. All the 395 strains were resistant to penicillin and 5.3 per cent also to tetracycline.

J. BÖSZÖRMÉNYI, J. SZITA

(Institute for Serobacteriological Production and Research "Human" and
National Institute of Public Health, Budapest)

PROPERTIES OF STREPTOCOCCUS STRAINS ISOLATED FROM ERYSIPELAS AND OTHER SKIN AFFECTIONS

From 329 patients with skin affections a total of 66 B-haemolytic streptococci strains was isolated, 21 from 115 erysipelas cases and 45 from 214 cases of eczema and pyoderma. Twenty-five of the isolates belonged to group A, 22 to group C and 19 to group G.

Fifty-five of the 66 strains were non-pathogenic for albino mice and the remaining 11 killed them only up to a dilution of 1:100. In contrast, 5 of 13 strains isolated for control from patients with scarlet fever and tonsillitis were pathogenic for mice up to a dilution of 1:10,000.

Patients infected with group G. streptococci had notably higher anti-streptokinase and antistreptolysin titres than the others.

The theory is advanced that erysipelas and other streptococcal skin infections involving the subcutaneous connective tissue and lymph vessels are caused by strains forming very little or no type specific M antigen. Thus they are non-pathogenic for mice, do not confer a durable type specific protection and allow for the frequent recurrence of erysipelas if there is a local predisposition.

F. KEMENES

(Department of Microbiology, University Veterinary School, Budapest)

ADAPTATION OF LEPTOSPIRA POMONA STRAINS ISOLATED FROM SWINE TO ALBINO MICE AND ALBINO RATS

In Hungary, *Leptospira pomona* has been the most frequent leptospiral serotype. Direct transmission of *L. pomona* strains isolated from swine, calf, horse or man to laboratory rodents is difficult as albino mice and rats do not become leptospira carriers. *L. pomona* does not occur in Hungarian wild rodent populations, either. Last year, however, *L. pomona* strains, readily transmissible to laboratory rodents, were isolated from a large breed of albino rats in Budapest. In view of this a *L. pomona* strain isolated from swine was carried through 20—30 serial passages in guinea pigs, using blood taken during the febrile course at approximately weekly intervals. Blood samples taken during the febrile course of leptospiraemia in the 20. — 30. passage were then inoculated into mice and rats. In the kidneys of the rats the pomona-type leptospiras were demonstrable for more than a year and their adaptation ability was identical with that of *L. pomona* isolated from the albino rats. Accordingly, *L. pomona* from swine, after an appropriate number of passages in guinea-pigs, appears to be capable of colonizing in the kidney of rodents.

F. KEMENES, GY. KUCSERA

(Department of Microbiology, Veterinary University School, and Control Institute for Veterinary Vaccines, Budapest)

A NEW METHOD FOR THE DEMONSTRATION OF H₂S PRODUCTION BY ERYSIPELOTHRIX RHUSIOPATHIAE

It has been shown that *Erysipelothrix rhusiopathiae* strains produce H₂S, while *Listeria monocytogenes* strains do not. So far the H₂S production by *E*

rhusiopathiae has been examined in agar cultures containing lead acetate and evaluated as negative or positive. In the present study a semi-liquid medium, containing nutrient broth with 0.2 per cent agar-agar, 1.0 per cent glucose, 1.5 per cent sodium thiosulfate, 0.5 per cent lead acetate and 10.0 per cent horse serum was used. In this medium the blackening took 48 hours only with all the 205 *E. rhusiopathiae* strains examined (148 type-A ones including 3 type-A₂; 33 type-B, 1 type-C, 1 type-D, 1 type-E and 20 type-N strains). In the same medium, 25 *L. monocytogenes* strains (21 type-I, 1 type-II, 1 type-III, 1 type-IVa and 1 type-IVb ones) showed no H₂S production. This indicates that H₂S production is a characteristic feature of bacteria belonging to the genus of *E. rhusiopathiae*. Further examinations are in progress to get a closer approach to the pathogenesis of chronic swine erysipelas.

M. RÓDLER, V. VADON

(Public Health Station of County Tolna, Szekszárd)

EXAMINATION OF H₂S PRODUCTION BY BACTERIA

H₂S production of different bacteria was examined in double-layer agar plate test, using cysteine and cystine as organic, and sodium thiosulfate as inorganic sulphur source. No notable difference was demonstrable in the sensitivity of lead acetate and ferrous salts as indicators of the produced H₂S.

A total of 119 bacterial strains were examined, belonging to the enteric and pigment producing species. All H₂S producing strains gave positive results with lead acetate as well as with iron salts in media with either organic or inorganic sulphur sources. In a medium containing 0.1 per cent cysteine, all H₂S-negative bacteria gave a positive reaction, with iron salts, however, only the *E. coli* strains were positive. No positive tests occurred in media with inorganic sulphur source.

Strains giving aspecific positive H₂S reactions in double layer media containing 2 per cent Bactoor Proteose peptone and tested with lead acetate were examined also in media supplemented with some extra organic sulphur source and tested with lead acetate as well as with iron salts. No positive reactions were observed with iron salts in media supplemented with 0.1 per cent cysteine, while positive reactions were obtained with lead acetate.

A total of 123 non-enteric bacterial strains were also tested. The pasteur-ella and listeria strains behaved like H₂S-negative organisms, giving a positive reaction with lead acetate in media supplemented with cysteine. Erysipelothrix strains were H₂S-positive, giving each positive reaction with lead acetate as well as with iron salts, in media containing either organic or inorganic sulphur. A variety of other H₂S-negative bacteria gave also positive results with lead acetate in cysteine-containing media.

However intensive a reaction was obtained with lead acetate in cystine or cysteine containing media inoculated with H_2S negative organisms, the reaction with iron salts remained invariably negative. This observation pointed to the possibility that the reaction with lead acetate was due to the presence of mercaptanes produced on decarboxylation or desamination of the cysteine molecule.

With several bacterial strains, the presence of mercaptanes was demonstrable by the nitroaryl-mercaptane test.

In the presence of organic sulphur, brucella strains behaved like H_2S -negative bacteria, reacting exclusively with lead acetate. The feeble positive reaction with lead acetate in sodium thiosulphate containing medium was supposed to be the result of a reaction with some non-volatile reducing substances produced by the bacteria.

The fact that lead acetate has been considered a sensitive indicator of H_2S production as compared to iron salts, is explained by the results of this study. Mercaptanes are produced from cystine contained by peptone preparations of satisfactory quality and they react readily with lead acetate. The appearance of this reaction precedes that given with iron salts. Aspecific positive reactions may also be caused by some difference in the amount of the available sulphur or by some minor differences in the bacterial enzyme activity.

It is concluded that the reaction demonstrable also with iron salts should only be considered a sign of H_2S production.

J. TAKÁCS

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CHARACTERISTICS OF LYSINE-DECARBOXYLASE ACTIVITY OF SALMONELLAE CAUSING SPECIFIC ANIMAL PARATYPHOID

The slowly growing salmonellae causing specific animal paratyphoid are the *S. cholerae-suis* var. *Kunzendorf*, *S. cholerae-suis* var. *America*, *S. typhi-suis*, *S. gallinarum-pullorum*, *S. abortus-equi*, *S. abortus-ovis*. Among these all the examined *S. typhi-suis* and 40 per cent of the *S. abortus-equi* strains gave a negative lysine-decarboxylase (LDC) test. On the contrary, all the *S. cholerae-suis* var. *Kunzendorf* and var. *America*, *S. abortus-ovis* and *S. gallinarum-pullorum* strains were positive in the lysine-decarboxylase test. The test was performed in a modified medium, using the CARLQUIST (1956) and SCHAAL (1962) ninhydrine test. The medium contained as lysine source HCl-hydrolysed casein instead of trypticase, casitone or casein hydrolysate. In the test, the dissimilar behaviour of salmonellae causing specific animal paratyphoid (*S. typhi-suis* and *S. abortus-equi* strains) must not be disregarded, in contrast with the uniformly LDC-positive salmonellae responsible for gastro-enteritis.

Zs. PUSZTAI, I. JOÓ, É. G. ECKHARDT, S. JASZOVSKY

(Institute for Serobacteriological Production and Research "Human", Budapest)

INFLUENCE OF NICOTINIC ACID ON THE ANTIGENIC STRUCTURE OF
BORDETELLA PERTUSSIS

Nicotinic acid, one of the essential growth factors for *Bordetella pertussis* has been used in increasing amounts (0.0005—0.05%) in shaken fluid cultures inoculated with several phase I strains. By this treatment changes have been observed in the morphological characters and in the capacity of multiplication. The inability to grow on nutrient or blood agar remained unchanged, but the cultures lost their agglutinability, mouse-protective potency and histamine-sensitizing ability by retaining their full toxicity. The loss of these properties run parallel with the applied quantity of nicotinic acid. Transferring the nicotinic acid treated bacteria into optimal media they regained all their immunobiological characters which were transiently lost. Immunochemical investigations (immunoelectrophoresis, enzymatic treatment, etc) proved that these alterations are probably consequences of an altered cell-wall synthesis.

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SOME FACTORS INFLUENCING THE TOXIN PRODUCTION OF
CL. TETANI IN PEPSIN-TRYPSIN DIGESTED BOVINE HEART BROTH

On the basis of extensive comparative studies of strains obtained from different institutes, *Cl. tetani* Harvard (Copenhagen) was found to be the most suitable for large-scale production of tetanus toxins.

During the cultivation period samples were taken at appropriate intervals for the determination of changes in alpha-amino nitrogen, iron, live germ count, turbidity and pH. The amount of toxin produced was estimated by the determination of MLD, L₊, Lf.

The effect on toxin production of iron and active carbon at different concentrations has been studied.

Strain *Cl. tetani* Harvard (Copenhagen) produced 50—55 L₊/ml toxin in pepsin-trypsin digested bovine heart broth medium supplemented with 150 mg Fe^{II}/ml and 3 g/litre active carbon. The presence of iron reduced the medium's oxidation-reduction potential, whereas the carbon adsorbed the inhibitors from the heart broth and the acid products which had formed during cultivation.

Identical amounts of toxin were produced in media pretreated with 10g/litre active carbon, provided the pH was maintained in the region of 7 during the period of cultivation.

I. RÉDAI, A RÉTHY

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FATTY ACIDS OF STAPHYLOCOCCUS AUREUS STRAINS OF DIFFERENT ANTIBIOTIC SENSITIVITY

The total fatty acid content and fatty acid spectrum was examined of a total of 10 *Staphylococcus aureus* strains of different antibiotic sensitivity. No notable differences in total fatty acid content were observed between antibiotic sensitive and resistant strains. Strains of different antibiotic sensitivity synthesize identical fatty acids, the main components of the fatty acid spectrum being anteiso-C₁₅, C₂₀, C₁₇ cyclopropane ring containing or branching fatty acids and stearic acid.

The different fatty acids isolated from strains of different antibiotic sensitivity, cultured 18 hours under strictly identical conditions, showed a different percentual distribution. Fatty acids containing cyclopropane ring or having a branching C-backbone represented 16 to 20 per cent and 34 to 44 per cent of the total fatty acids of antibiotic sensitive and resistant strains, respectively.

The strains were found to synthesize branching or cyclopropane ring containing fatty acids in semi-synthetic media containing Tripcasin ("Human", Budapest), vitamin B₁ and nicotinic acid amide.

M. BOJÁN

(Public Health Station of County Hajdú-Bihar, Debrecen)

SENSITIVITY TO HEAT OF COAGULASE POSITIVE STAPHYLOCOCCI ISOLATED FROM FOODSTUFFS

The sensitivity to heat of coagulase positive *Staphylococcus aureus* strains isolated from foodstuffs having caused food poisoning was 20 per cent higher than that of other similar strains isolated routinely from various foodstuffs and from purity tests. The difference should be considered significant. The difference in heat tolerance of the strains decreased when the temperature approached the lethal degree.

Two-thirds of thermoresistant pathogenic strains, while only 30 per cent of the non-pathogenic strains of low thermoresistance belonged to phage type 3. This observation is in agreement with the literary data on the high incidence of phage type 3 staphylococci in food poisonings.

M. LAKATOS

(Public Health Station of County Hajdú-Bihar, Debrecen)

DIAGNOSTIC VALUE OF DIFFERENT SEROLOGICAL TESTS IN HUMAN BRUCELLOSIS

Considering the highly variable clinical appearance of brucellosis the results of laboratory examinations may often be of decisive importance. Cultivation of the organisms often fails, thus particular importance should be attached to the serological tests.

Blood samples of 476 persons working with cattle were subjected to the WRIGHT's agglutination reaction, COOMBS' test and complement fixation (CF) reaction. Out of these agglutination yielded the lowest incidence of positivity (10 per cent). With COOMBS' test, the incidence of positive reactions was 31.1 per cent. The CF reaction was strongly positive in 12.4 per cent and weakly positive in 10.1 per cent.

COOMBS' test, serving for the demonstration of incomplete antibodies, and the CF reaction are of particular importance in the diagnosis of chronic brucellosis and in the late diagnostics of the disease.

The intradermal test performed with KOLLÁR's F-allergen proved to be an appropriate supplementary test, as it exhibited clear-cut positive reactions in 22 per cent and a weak positive reaction in 14.2 per cent of the cases. This test was always positive in chronic cases and sometimes also in those proving negative in the other serological test.

The simultaneous use of all the four tests mentioned ensured a positive reaction for 182 (38.2 per cent) of the 476 persons examined.

M. LAKATOS

(Public Health Station of County Hajdú-Bihar, Debrecen)

INCIDENCE OF BRUCELLOSIS IN HAJDÚ-BIHAR COUNTY COW FARMS ON THE BASIS OF MILK EXAMINATION

The ABR or ring test performed with milk may be employed for estimating the incidence of brucellosis in a given cattle stock. In 15 cow farms, milk samples from a total of 1920 cows were examined. The test was positive in 23.3 per cent, weakly positive in 10.4 per cent. In cow herds where abortion due to acute brucellosis was frequent, the number of strongly positive reactions was usually higher than the average. The 72 milk samples giving a positive ring test were inoculated into albino mice. Five strains were isolated.

Twenty positive milk samples were inoculated into guinea-pigs in order to demonstrate the presence of brucellae by the agglutinin titre increase in

these animals. In five animals reactions were obtained that could be considered positive. Four animals died one day after the inoculation and brucellae were isolated from their spleens. The isolated strains belonged to the type *Brucella bovis*.

E. FÁBIÁN, J. KISS

(Department of Tuberculosis, University Medical School, Debrecen)

DYNAMISM OF THE BACTERIAL FLORA OF CAVERNOSTOMY CAVITIES

The general bacterial and the TB flora of cavities subjected to open treatment has been examined, registering the changes in composition and resistance of the organisms. Out of 996 routine bacteriological examinations, 218 (21.9 per cent) were negative. Among the 778 positive cultures the incidence of *Ps. pyocyaneus*, *P. vulgaris* and *St. aureus* was 32.1, 22.5 and 22.5 per cent, respectively. Thus, 77.1 per cent of the isolates belonged to these three species. The incidence of these three bacterium species in repeated cultures was studied and the problems of antibacterial treatment and resistance were discussed. The possibility of isolating *M. tuberculosis* was practically identical in Loewenstein-Jensen and in slide cultures. No correlation was found between the common and TB bacterial flora of the residual cavity.

The average duration of open treatment was 138 days. Such a prolonged treatment involves the risks of hospitalism and of an increase in resistance, and makes it difficult to protect the other wards from the infection. These cases require the closest cooperation between the laboratory and the surgical section.

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and Department of Paediatrics, University Medical School, Debrecen)

SENSITIVITY TO METHICILLIN AND OXACILLIN OF STAPHYLOCOCCUS AUREUS STRAINS ISOLATED FROM HOSPITAL STAFF

Sensitivity to methicillin and oxacillin of 66 and 53 *St. aureus* strains isolated from staff members of the Department of Obstetrics and the Department of Paediatrics, respectively, was compared. At a concentration of 8 µg/ml, methicillin inhibited 2 per cent and 28 per cent of the strains of gynaecological, respectively paediatric origin. These strains have been considered methicillin tolerant. No methicillin resistant strain was isolated.

At concentrations of 4 µg/ml and 2 µg/ml, oxacillin inhibited 1 and 9 per cent of the gynaecological, and 8 and 26 per cent of the paediatric strains, respectively.

The different sensitivity to methicillin and oxacillin of the *St. aureus* strains isolated in the two departments is explained by differences in their penicillinase production and resistance to other antibiotics, due most probably to the use of different antibiotics in the two institutions.

Among the gynaecological strains 3 per cent were inhibited at a penicillin concentration as high as 400 $\mu\text{g/ml}$, and only 9 and 5 per cent of them were resistant to 5 and 6 antibiotics, respectively. Among the paediatric strains, however, 26 per cent required 400 $\mu\text{g/ml}$ of penicillin for inhibition, and 15 and 30 per cent were resistant to 5 and 6 antibiotics, respectively.

The greater incidence of methicillin tolerant strains among the paediatric staff might have been due also to the fact that at the Paediatric Department methicillin has been used regularly for 4 years.

IMMUNOLOGY

K. RAUSS, I. KÉTYI, T. ANGYAL

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IMMUNOGENICITY OF VACCINES PREPARED FROM LIVE AVIRULENT SHIGELLA STRAINS

Antigenically intact, avirulent mutants of *Sh. flexneri* types 2a and 3 have been selected on the basis of light refraction of their colonies. The avirulent and *E. coli* $\times$ *Shigella* hybrid strain of FORMAL *et al.* and the streptomycin dependent strain used with success for oral immunization by MEL *et al.* have been included in the study. Based on their infectivity for the mouse and their behaviour in intravenous and allantoic chick embryo test, the mutants were divided into three groups, *viz.* those which have lost the ability (i) to penetrate, (ii) to intratissular multiplication, and (iii) to both.

The mutants (i) and (iii) were equivalent in immunogenicity to killed germs and inferior to the Boivin extract. The immunogenic effect of mutants (ii), inhibited only in tissular multiplication, exceeded that of killed vaccines by one logarithmic exponent and they were equivalent in antigenicity with the Boivin extract.

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PROLONGATION BY ORAL IMMUNIZATION OF THE IMMUNITY CONFERRED BY PRECIPITATED PARENTERAL VACCINE

Subjects vaccinated twice with, and given a booster dose of, precipitated Typhoid-Dysentery-Tetanus combined parenteral vaccine, were treated

with oral vaccine once weekly for 6–12 weeks. The tablets used for oral vaccination were prepared of the same three components as the parenteral vaccine, but the antigenic value of each component was five-fold of that applied in the parenteral dose. The antigens were concentrated by precipitation with alcohol. The control group received vaccine parenterally, but placebo tablets for oral treatment.

Pooled sera from blood samples taken at different points of time showed a markedly decreasing tendency of mouse protective antibodies 3 months after parenteral vaccination in the control group, while in the orally vaccinated group these antibodies remained at the original high level throughout the period of revaccination. This means that revaccination by the oral route would substantially prolong the immunity to dysentery.

I. KÉTYI, K. RAUSS

(*Institute of Microbiology, University Medical School, Pécs*)

ADJUVANT EFFECT OF STREPTOMYCIN ON ORAL VACCINATION

It has been shown earlier that the infectivity of virulent *Shigella* strains for the mouse may be increased by 4–5 exponents by pretreatment with streptomycin. In view of this finding, the antigenicity enhancing effect of streptomycin on oral vaccination was examined. With strains able to penetrate the intestinal mucosa but inhibited in multiplication in tissues, streptomycin increased the immunogenicity by about 4 exponents. With fully avirulent strains this increase amounted to 2–3 exponents, similarly to formol-killed vaccines. With Boivin-type extracts, antigenicity could be increased by approximately 4 exponents, *viz.* to the same extent as with the live mitigated strains.

The mechanism of the adjuvant action of streptomycin is based presumably on the enhancement of intestinal permeability.

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PRINCIPLES AND SOME APPLICATIONS OF THE IMMUNOGRAM

Using a serological micro-method, the presence and titre of antibodies in human sera have been determined against about 30 different environmental antigens. Antibodies to bacterial antigens, proteotoxins and red blood cell antigens were studied. Titres of 13 antibodies present in about 80 per cent of normal human sera were examined in patient sera. *Staphylococcus* alpha antitoxin, antibodies to *Salmonella typhi* 0 and Vi antigens were IgG and IgA-

type immune globulins. Antibodies to *Sh. flexneri* 2a were IgA, while those to *E. coli* 026, 055 and *Sh. flexneri* 1b, IgA and IgM immune globulins. Antibodies to *Sh. flexneri* 3, *E. coli* 086, 0111 were IgM, whereas the anti-A, anti-B isohaemagglutinins and antibodies to *Sh. sonnei* were IgA, IgM and IgG immune globulins. The average antibody titres referred to the normal value are presented in a graph which we call immunogram. Groups of diseases were examined which were known to influence the production of antibodies. In the serum of patients with agammaglobulinaemia, dysgammaglobulinaemia or paraproteinaemia of IgG or IgM type, a considerable decrease in the antibody titre was observed. A moderate decrease was demonstrable in the sera of patients with paraproteinaemias and malignant haemoblastoses. Elevated antibody titres were present in vegetative and organic hyperthyreoses. Average values in patients with cirrhosis were similar to the normal ones, but some patients exhibited considerably higher, while others considerably lower titres.

For estimation of immunological reactivity the immunogram is superior to active immunization with one or several antigens.

I. BERCZI, K. BAINTEK, Jr., T. ANTAL, L. BERTÓK

(Research Institute of Veterinary Medicine, Hungarian Academy of Sciences and
Research Institute of Veterinary Physiology, Budapest)

ABSORPTION OF ENDOTOXINS FROM THE INTESTINE

The following three types of absorption have been studied in different animal species. I. Macromolecular absorption absent: adult rat and mouse; II. Selective macromolecular absorption: suckling rats younger than 21 days; III. Non-selective macromolecular absorption: newborn pig and calf. The endotoxins used in the experiments were prepared from *E. coli* strains, using the warm phenol-water method of WESTPHAL *et al.*

Oral administration of an endotoxin dose corresponding to DL_{100} (100 mg/kg for rats and mice, 5 mg/kg for the pig and 100 μ g/kg for the calf) caused no clinical symptoms whatever in the experimental animals. Similar results were obtained when 5–10fold amounts of the above doses were applied orally to suckling rats, newborn pigs and calves.

Rats rendered sensitive to endotoxin by intravenous treatment with 50 mg/kg lead acetate were given 3000 DL_{100} (100 mg/kg) endotoxin orally, simultaneously with, and 3 and 6 hours before and after, the administration of lead acetate. These animals, too, remained free of clinical symptoms. The results are discussed with respect of endotoxin absorption from the intestines.

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Budapest)

STUDIES ON RATS HYPERSENSITIZED TO ENDOTOXIN WITH LEAD
ACETATE

Previous studies by SELYE, TUCHWEBER and BERTÓK (J. Bact. 91, 884, 1966) have shown that intravenously administered subtoxic doses (5 mg/100 g body weight) of lead acetate may extremely (10,000–100,000-fold) increase the rat's sensitivity to endotoxin. The same effect was observed with purified endotoxin and with small intravenous amounts of live germs, for example, 1 ml of 10^{-4} diluted 24-hour broth cultures of *E. coli* or *Proteus vulgaris*. Lead acetate caused a prolongation of experimentally induced bacteraemia. The hypersensitivity to endotoxin induced by lead acetate may be reduced by the simultaneous administration of some compounds rich in SH groups (cysteine-HCl, glutathione, etc.). Thus the joint action of lead acetate (5 mg) and endotoxin (1–3 μ g), causing in every case 100 per cent mortality, was reduced to 20, 40 and 60 per cent by cysteine HCl, glutathione and methionine, respectively. According to the working hypothesis evolved by the authors, the SH groups (adaptive enzymes?) present in the organism may play a substantial role in the detoxication of endotoxin. It has been shown that the so-called endotoxin tolerance, elicited by pretreatment with small doses of endotoxin or bacteria, would confer a firm protection against the hypersensitivity to endotoxin induced by lead acetate.

I. BERCZI

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Budapest)

DEMONSTRATION OF THE ENDOTOXIN NEUTRALIZING EFFECT OF
SPECIFIC IMMUNE SERA IN THE CHICK EMBRYO

Lipopolysaccharide endotoxin was extracted from *E. coli* 078 by the method of WESTPHAL *et al.* and absorbed to casein according to TAKEDA *et al.* Hyperimmune sera were prepared in rabbits with a mixture of lipopolysaccharide casein complex antigen and complete Freund adjuvant (Difco). On incubation at 37°C, for 1 hour, 0.005 ml hyperimmune serum neutralized the 100fold chick embryo lethal dose (DL_{100} —0.005 μ g/embryo) of the homologous endotoxin.

T. G. KOVÁTS, P. VÉGH

(*Institute of Pharmacology, University Medical School, Szeged*)

ENDOTOXIN HYPERSENSITIVITY AND IMMUNE-SPECIFICITY

On the basis of previous results it has been supposed that besides primary toxic effects an immune-specific reaction is playing a role in the first stage — the local endotoxin hypersensitivity — of the Shwartzman phenomenon. An attempt was made to confirm the immune-specificity on the basis of endotoxin resistance. In rabbits rendered moderately resistant by *E. coli* or serratia endotoxin, preparation and provocation of the phenomenon was attempted partly with homologous, partly with heterologous endotoxin.

The animals resistant to *E. coli* endotoxin did not display a Shwartzman reaction when prepared intradermally with *E. coli* endotoxin, but exhibited distinct Shwartzman phenomena at the sites prepared with serratia or typhoid endotoxin.

Rabbits rendered resistant to serratia endotoxin did not show the reaction when prepared intradermally with homologous serratia endotoxin, but reacted with an intensive haemorrhagic reaction to cutaneous preparation by heterologous endotoxins.

These results indicate the involvement of an immune-specific reaction in the cutaneous preparation for the Shwartzman phenomenon. Intravenous provocation, however, may uniformly be carried out with homologous or heterologous endotoxin, thus no immune-specific factors appear to play a role in this stage.

In animals with high endotoxin resistance, immune-specificity is observable only when either the preparatory, or the provoking dose is increased.

P. VÉGH, T. G. KOVÁTS

(*Institute of Pharmacology, University Medical School, Szeged*)

SEASONAL VARIATIONS OF ENDOTOXIN SENSITIVITY

In previous studies on endotoxin sensitivity and immuno-specificity, using the Shwartzman reaction as a model, notable changes were observed in sensitivity and resistance to endotoxin. In experiments performed in the months January and June, five small intravenous doses of endotoxin were required to induce a moderate resistance in rabbits. In October the animals displayed a very weak Shwartzman reactivity and two small doses of endotoxin induced such a high degree of resistance that no Shwartzman phenomenon could be provoked either with homologous or with heterologous endotoxin. This indicates that in the autumn the animals develop spontaneously a high degree of resistance. In the endotoxin resistance induced by two endotoxin doses, im-

mune-specificity was also demonstrable, but exclusively in cases when either the preparatory or the provoking endotoxin dose was increased.

The seasonal fluctuations demonstrable in the sensitivity (or resistance) of animals to endotoxin are explained by the assumption that the stress imposed by the warm summer may have influenced the non-specific defence mechanisms, *e.g.* the reticulo-endothelial and endocrine systems.

L. RÉTHY

(Institute for Serobacteriological Production and Research "Human", Budapest)

FURTHER STUDIES ON ADJUVATING ACTION OF ENDOTOXINS

Previous studies have revealed a well-defined relationship between the adjuvant effect and quantity, origin, and direct pharmacological action of endotoxins. Also, adjuvant effect of endotoxins on "priming" and "secondary stimulus" has been examined. In both cases positive results were obtained in field experiments on humans and in laboratory trials on animals.

In the present study, the effect of endotoxins in "booster antigenic stimulus" has been investigated. It was found that the adjuvant effect of endotoxins manifests itself also at booster doses.

The quantitative relationships of endotoxin-dependent adjuvancy have repeatedly been studied in model experiments, by comparison with the adjuvant action of quaternary ammonium bases.

In relation to the immunogenicity of tetanus antigen, the quaternary ammonium bases proved to be superior adjuvants, but their action mechanism differed distinctly from that of the endotoxins.

L. RÉTHY, L. HEGEDÜS, G. TÓTH, V. P. JUHÁSZ

(Institute for Serobacteriological Production and "Research Human", Budapest)

MODEL EXPERIMENTS ON THE COMPETITION OF ANTIGENS

The antigenic competition between diphtheria and tetanus toxoids has been studied in guinea pigs and in a modified mouse test. Diphtheria antigen had an inhibitory effect on the efficacy of tetanus toxoid although according to the hierarchy of antigens, the former was the "weaker" and the latter the "stronger". The degree of inhibition depended on the protein quota of associated diphtheria antigen, *i.e.* on the ratio mg diphtheria PN/mg tetanus PN.

The competition of antigens was not always demonstrable in guinea pigs, while in the other immunological test systems the inhibition was obvious.

No inhibitory effect was demonstrable while administering the competing antigens with endotoxin-containing adjuvant.

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ROLE OF COLOSTRUM IN *E. COLI*-TOXICOSIS

In Hungary the *E. coli* diseases of newborn pigs and calves occur as a rule without septicaemia, but with toxic signs. When, however, calves kept in infected environment were deprived of colostrum and given normal cow's milk instead, the *E. coli* strains gave rise to septicaemia. One hundred and twenty five samples of colostrum from a herd where *E. coli*-toxycosis was frequent among the calves, were examined with antigens of *E. coli* strains isolated from the same herd, by agar gel diffusion and passive haemagglutination tests. Specific antibodies were demonstrable in 56 and 100 per cent of the samples by the gel diffusion and passive haemagglutination tests, respectively. The important role of specific antibodies in the manifestation of the disease was also confirmed by experiments with specific hyperimmune sera. It is believed that the manifestation of *E. coli*-disease in a toxic or septicaemic form depends beside the pathogenicity of the involved *E. coli* strain also on the colostral immunity of the newborn. Animals with high colostral immunity resist infection by pathogenic *E. coli*, those with medium protection develop a varying degree of diarrhoea or toxicosis, and those with low or no colostral immunity develop the septicaemic form.

T. ANGYAL

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EXAMINATION OF PAIRED SERA AFTER STAPHYLOCOCCUS VACCINATION

Forty-nine patients suffering from chronic or relapsing staphylococcal dermatitis were treated with autovaccine, commercial polyvalent vaccine and vaccines prepared of both diffuse and compact variants of the Smith strain. The autovaccine and the Smith "diffuse" vaccine showed a remarkable therapeutic effect. The clinical effectiveness was supported by the enhanced phagocyte index and by the mouse protection test. In the serum samples of vaccinated persons the rate of phagocytosis rose by about 40 per cent during the first 15 minutes. The sera of the same persons protected mice in a quantity of 1.5×10^{-4} ml against 20 LD₅₀ of the Smith diffuse variant. Titre values in the haemagglutination test did not exceed 1 : 127 but in Coombs' haemagglutination test the titre was 2048. These titres, however, did not indicate the clinically proven immunity, since sera taken before immunization showed the same titres. The changes on alpha antitoxin value remained in the normal range, the average value after vaccination was 1.71 T.U.

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EFFECT OF H-PERTUSSIS VACCINE (HPV) ON THE HISTAMINE AND
5-HYDROXYTRYPTAMINE (5-HT) CONTENT OF RAT AND MOUSE TISSUES

On inoculation of HPV into rats, the histamine level increased markedly in their blood, liver and lung, but decreased in the pylorus and skin. The 5-HT level increased only in the stomach and blood, decreased markedly in the liver, and moderately but significantly in the skin. The 5-HIAA and free histamine uniformly tended to increase, the latter to about 100fold, reaching the peak by the 8th day. In both animal species, histamine activity of the tissues decreased considerably on the 1st day, as compared to the controls. As the change of the amine level and the decrease of histaminase activity elicited by HPV were similar to those induced by adrenalectomy, it is supposed that HPV develops its action through the adrenals.

I. NYIREDY, L. HÉJJ, S. TUBOLY

(Central Veterinary Institute, Budapest)

THE ROLE OF SAPROPHYTIC MYCOBACTERIA IN ELICITING
TUBERCULIN SENSITIVITY IN CATTLE

Tuberculosis-free 3—6 months old calves were infected orally on 7 occasions with *Mycobacterium phlei*, *M. smegmatis*, *M. butyricum*, *M. pellegrino* (5 calves each) or *M. minetti* (42 calves). Subcutaneous infection on two occasions was made with *M. fortuitum* and *M. minetti* in 3 and 2 calves, respectively. The 20 animals infected with the first four strains gave negative reaction to mammalian, avian, *phlei* and *minetti* tuberculin. In the 42 calves infected orally with the *M. minetti* strain, testing with mammalian, avian and *minetti* tuberculin on 3 occasions had the following results. A positive reaction was obtained in 66.6 per cent to the homologous tuberculin, in 19 per cent to mammalian tuberculin, in 12 per cent to avian tuberculin. Simultaneous positive reaction to all the three allergens was exhibited by 4.8 per cent, to mammalian and homologous tuberculin by 12 per cent, to avian and *minetti* tuberculin by 2.4 per cent. Reaction to mammalian tuberculin alone was 2.4 per cent, to avian alone 4.8 per cent. Among the three animals infected subcutaneously with *M. fortuitum*, all reacted positively to avian tuberculin and one of them also to mammalian tuberculin. Out of the two calves infected subcutaneously with *M. minetti*, one reacted only to avian, the other only to mammalian tuberculin. In both of them, intracutaneous injection of the homologous allergen resulted in doubtful reaction. This indicates that the oral ingestion of

some saprophytic mycobacteria may evoke in part of the animals a strong immune response with positive or doubtful reactions to mammalian and/or avian tuberculin even several months after the infections has ceased.

The immune response to *M. minetti* exhibited some individual variations; this might have been due to the predisposing factors or defects in the digestive tract.

The results suggest that the strain *M. minetti* is more easily adaptable to cattle than the other saprophytic mycobacteria included in this study. Also, the degree of immune response to mammalian and avian tuberculin seems to depend largely on the number and quantitative relationships of common antigenic fractions in the pathogenic and saprophytic mycobacteria.

The conclusion is drawn that the reaction known as paraallergic is invariably specific, being brought about by cross reactions due to the common antigenic components of the different mycobacterium species. Therefore, instead of paraallergy the more precise term *mycobacterioallergy* is proposed.

L. HÉJJ

(Central Veterinary Institute, Budapest)

INFLUENCE OF REPEATED INTRADERMAL TESTS ON ALLERGIC DISPOSITION

After performance of intradermal tuberculin test on 567 tuberculous cattle, the animals were divided into 9 groups (44—84 animals per group) and injected again intradermally with tuberculin. The first group was treated on the very day of the first reading and the last one 8 days later.

Out of the 567 animals, all positive at the first reading, 28 showed a doubtful and 30 a negative reaction at the second reading. Swelling around the reaction was considerably reduced. While after the first reading the average thickening of the skin was 12.2 mm, after the second one it averaged 8.2 mm (67.2 per cent); it was lowest in the 4th group (54 per cent).

This shows that in tuberculous cattle, repeated intradermal tuberculin tests should be evaluated with caution at least up to the 9th day after the first testing, as the results may fall under the limit of positivity by as much as 67.2 per cent, thus interfering with the detection of the infected animals.

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(Institute for Serobacteriological Production and Research "Human", Budapest)

STANDARDIZATION OF TETANUS ANTIGEN

Estimation of tetanus toxoid immunogenicity has been carried out by graded immunization of mice and subsequent toxin challenge with the method by IPSEN, GREENBERG and VAN RAMSHORT.

The reproducibility of the method was found to be superior to that of guinea-pig tests. The optimal timing of immunization and challenge was determined.

The efficiency of the method was compared with that of guinea-pig immunization tests. The mouse test was found to allow a more precise standardization of tetanus toxoid than the guinea-pig one- or two-stimulant method.

Using the proposed standard WHO tetanus toxoid, a home substandard preparation was developed.

K. MÉRETEI, L. KOCSÁR, E. ELEKES

("Frédéric Joliot-Curie" Research Institute for Radiobiology and Radiohygiene, Budapest)

SOLUBLE TISSUE-ANTIGENS IN GUINEA-PIG LIVER

Guinea-pig liver homogenates and subcellular fractions obtained by ultracentrifugation have been studied by agar electrophoresis and subsequent staining for protein, fat and nucleic acids. One to 7 fractions could be demonstrated with protein staining and 0 to 3 fractions with fat and nucleic acid staining. Absorbed rabbit serum acting against the liver homogenate yielded 3 to 9 precipitation lines with the examined antigens. While the mitochondrial, microsome and hyaloplasmic fractions contained 7 to 8 common antigens, the antigen pattern of the deoxycholate-soluble microsome fraction was different. The demonstrated antigens proved to be species-specific. Common antigens were found both in the spleen and in the liver. Immune serum failed to give a precipitation reaction with a ribonucleoprotein fraction extracted from the liver by chemical methods. On the other hand, the antiserum produced against RNA did react with the antigens present in the liver homogenate. Informative studies were done by the angular diffusion method to estimate the diffusion coefficient of the soluble liver antigens.

GY. LADA, R. BACKHAUSZ, GY. KERKOVICS

(Institute of Serobacteriological Production and Research "Human", and Bajcsy-Zsilinszky Hospital, Budapest)

IMMUNOCHEMICAL EXAMINATION OF SOLUBLE ANTIGENS OF THE HUMAN MYOCARDIUM

In conditions associated with tissue disintegration, antigens may be demonstrable in the circulating blood. Part of these antigens is specific for the organ involved, part of them, however, is nonspecific, being demonstrable in

some other organs. In the present study we examined the appearance of common tissue antigens in cases of cardiac infarction.

Rabbits were immunized with extracts of human heart tissue containing also some serum protein. The immune sera obtained were absorbed with human plasma protein. In the heart antigen solution using absorbed immune sera we demonstrated by means of a modified immunoelectrophoresis the presence of antigens having electrophoretic mobilities like to the alpha-0, alpha-1, alpha-2 and gamma globulins.

By means of radial immunodiffusion and immuno electrophoresis, a total of 25 sera from patients with acute myocardial infarction was examined; 23 contained a tissue antigen which was considered gamma-X globulin on the basis of its localization and antigenicity. This antigen was also present in sera from convalescent hepatitis patients.

HEREMANS and SCHULTZE were the first to describe gamma-X globulin, a protein of gamma globulin motility appearing in patients with some serious tissue disintegration. They thought it identical with CRP. However, the CRP prepared in this laboratory from patients who had died of cancer proved to be a protein of alpha-2 motility and did not seem to be identical with gamma-X globulin concerning its antigenicity.

We demonstrated in the sera from patients with myocardial infarction a protein of tissue origin and of gamma globulin motility. The protein was not specific for heart tissue and was demonstrable only with sera containing a precipitin against gamma-X globulin.

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EFFECT OF THYMUS MODEL ON ANTIBODY PRODUCTION IN ADULT ANIMALS

By implanting a homologous embryonic trachea into the spleen of 12 rabbits, 38 guinea-pigs and 76 rats, a so-called additional thymus model was created. According to TÖRŐ and CSABA this model, at least to some extent, acts as a thymus.

The specific immune response in guinea-pigs and rats with additional thymus model was about 100 per cent higher than in the controls. The difference was significant statistically.

This effect could be detected in rabbits, too. The effect exerted on antibody formation was, however, decidedly less explicit in rabbits than in guinea-pigs and rats.

Adopting JERNE's plaque method, it has been shown that with the increase in antibody level induced by the thymus model the number of anti-

body producing cells in the spleen increased about 5 to 6-fold, as compared to the controls.

The results prove the role of the thymus in antibody production not only in the initial phase of extrauterine life, but also in the adult, immunologically mature organism.

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ANTIBODY PRODUCING CELLS IN THE RAT SPLEEN

The antibody producing cells in rat spleen were studied by the plaque method described by JERNE in 1963. The decrease of plaque count in proportion to the dilution of spleen cell suspensions proved the proper application of the method. Each rat was treated intramuscularly with 0.1 ml of $\text{Al}(\text{OH})_3$ or 0.1 ml of incomplete FREUND's adjuvant containing no antigen. The spleens were worked up on the 5th, 11th and 17th days after treatment. The count of haemolysin-producing cells per 5×10^5 spleen cells was determined and the haemolysin and haemagglutinin titres of sera from the experimental animals were estimated. In the rats treated with $\text{Al}(\text{OH})_3$, a significant drop of the plaque count was found at all the three sampling intervals (6.61 plaques/ 5×10^5 spleen cells ± 5.54 ; 6.42 plaques/ 5×10^5 spleen cells ± 1.38 ; 3.30 plaques/ 5×10^5 spleen cells ± 1.51), as compared to the controls (15.3 plaques/ 5×10^5 spleen cells ± 7.57 , respectively). The groups treated with incomplete FREUND's adjuvant displayed no significant differences on the 5th day (14.97 plaques/ 5×10^5 spleen cells ± 7.21). On the other hand, significant differences were observed on the 11th and 17th days (2.22 plaques/ 5×10^5 spleen cells $\pm 4-14$; 6.58 plaques/ 5×10^5 spleen cells ± 3.04 , respectively). The relative drop in the count of antibody producing cells was not accompanied by changes in the haemolysin or haemagglutinin titres. In relation to body weight, spleen weight was higher during the test period. The changes have not been due to a local effect of the adjuvants.

B. CSABA

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EFFECT OF HYPOTHERMIA AND HYPERTHERMIA ON ANAPHYLAXIS IN THE RAT AND THE MOUSE

Seventy to 80 per cent of albino mice and rats sensitized with crystalline ovalbumin reacted with lethal anaphylactic shock to antigen reinjection when treated 4-5 days before shock induction with *H. pertussis* vaccine. In rats,

death could not be prevented either with hypothermic (20–22°C rectal temperature) or hyperthermic (immersion into 45°C water bath for 15 minutes) treatment; in the hypothermic state, death rate was 100 per cent. In mice, however, both kinds of treatment, and especially the hypothermic one (20–24°C rectal temperature) decreased the death rate from 70 to 51 per cent. In a 45°C water bath the mice died in 5–10 minutes, therefore hyperthermic treatment consisted of immersion in a 42°C water bath for 10 minutes. In the animals died of anaphylactic shock, similarly to the controls, severe haemorrhage involving the whole small intestine as well as enlargement and haemorrhage of the adrenals were detected. This indicates that the — presumably proteolytic — processes involved in the anaphylactic shock of the examined two species are not sensitive to heat.

The sensitized rats resisted the shock-inducing reinjection when half an hour earlier they had been treated with methylethylpyrimethine plus butazolidine, either with or without chlorpyramine. The contribution of chlorpyramine with ascorbic acid was also effective. The mediators of anaphylactic shock in rat and mouse seem therefore to be biologically active polypeptides, formed presumably as a result of proteolysis taking place in the small intestine. Nevertheless, 5-HT and histamine, released during the shock, are also playing a role in precipitating the shock.

6.

GY. DÓBIÁS, M. BALÁZS

(*Postgraduate Medical School, Budapest*)

PATHOGENIC ROLE OF HUMORAL ANTIBODIES REACTING WITH
SUBCELLULAR ORGAN ANTIGENS IN PASSIVE IMMUNIZATION

Sera from rabbits not immunized and immunized with mitochondrial fractions from rat liver and spleen were administered to rats intravenously or subcutaneously, at 3–4-day intervals on a total on 14 occasions. The total dose was 2.8 or 0.28 ml per animal and the period of observation lasted for 70 days. In comparison to the controls, rats treated with anti-liver sera exhibited increased bromosulphalein retention, perilobular fibrosis and, in one case, cirrhosis of the liver. Urinary protein excretion and histological structure of the kidneys did not differ from those observed in the control group. In animals treated with anti-kidney serum, the amount of protein excreted with urine over 24 hours was higher than in the control group, and in the renal glomeruli cell proliferation, in the tubules hydropic degeneration was detected, but bromo-sulphalein retention and hepatic structure did not differ from those observed in the control group. The extent of lesions appeared to depend on the dose and route of administration of the immune serum.

J. SURJÁN

(*Phylaxia State Serum Institute, Budapest*)

EXAMINATION OF IMMUNE GLOBULINS IN SERA FROM
SUCKLING PIGS

Protein fractions of swine sera were examined with rabbit serum prepared by means of immune-electrophoresis against swine serum. In sera from hyperimmunized adult swine, gamma globulin appeared in two fractions corresponding to IgG and IgA. There was also a strong typical IgM fraction. In sera from newborn pigs neither Ig nor IgM were demonstrable, but after the animals had had colostrum, both fractions appeared as distinct immune electrophoretic arcs. The temporal coincidence of their appearance with the establishment of colostrum immunity may be considered an indirect proof of their antibody nature. A direct proof was the specific precipitation by IgA and IgM of different bacterial antigens in the agar gel diffusion system.

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IMMUNO-ELECTRONMICROSCOPY OF HAEMAGGLUTINATION INDUCED
BY IODINATED COOMBS-SERUM

It is known that the electron density of iodinated proteins tends to increase and iodinated immune globulins thus become visible under the electron microscope. Anti human globulin rabbit serum (Coombs serum) and normal serum were treated with non-radioactive iodine salts, according to the method of BOURSNEILL, COOMBS and RIZK [1953]. In the direct Coombs test, Rh-positive human erythrocytes sensitized with anti-D serum were examined with iodinated and non-iodinated Coombs serum and iodinated normal serum. Agglutinates of the red blood cells treated with iodine-labelled immune serum, red blood cells treated with the normal iodinated control serum, and conglomerates of red blood cells treated with non-iodinated Coombs serum each were fixed in MILLONIG's buffered osmium tetroxide for one hour, washed with buffer solution, dehydrated with alcohol and embedded in Araldite. Ultrathin sections were cut with LKB or Tesla ultra-microtome and examined by a Tesla 242 D portable electron microscope. At 4 to 8 sites of the surface membranes of red blood cells treated with specific and iodine-labelled serum, electron-dense plaques of approximately 500 Å dimension were seen. Such plaques were not, or very sporadically apparent on red blood cells treated with iodine-labelled normal control serum. On the red blood cells treated with non-iodinated Coombs serum, no electron-dense plaques were present. These ex-

periments furnished a direct visible proof that in the examined systems the antigen-antibody linkage is taking place on the surface, indicated in the form of electron shadow owing to the high atom number (I) of the labelled antibody.

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PRESENCE OF BLOOD GROUP A ANTIGEN IN MEDIA FOR
CLOSTRIDIUM TETANI AND IN TETANUS ANTIGENS

The blood group A antigen content was examined by the haemagglutination inhibition test, performed with TAKÁTSY'S microtitrator, in tetanus toxin, native and concentrated toxoid, singular and combined vaccines obtained from *Cl. tetani* Harvard grown in enzymatically digested bovine heart broth.

The presence of blood group A substance was demonstrated in the meat extract obtained by digestion with either gastric mucosa of hogs or pepsin in the broth, the toxin and both the native and the concentrated toxoid. Digestion with trypsin or erepsin did not influence the titres.

About 50 per cent of the blood group A antigen was removed from the native toxoids by purification and concentration with trichloroacetic acid. Treatment with active carbon resulted in the elimination of blood group A antigen from both the native and the concentrated toxoid. The blood group substance was absent from the supernatants of vaccines containing tetanus antigen. The substance was not demonstrable in Mueller—Miller medium not containing any native protein.

J. SZATHMÁRY

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INHIBITION OF VACCINIAL HAEMAGGLUTINATION BY HYPERIMMUNE
ANTITOXIC HORSE-SERA

Sera of horses hyperimmunized with diphtheria, *Clostridium novyi*, streptococci, and tetanus toxoids and toxins were found to inhibit the haemagglutination of vaccinia virus at various titres. Studies involving a total of 195 horse antisera have shown that in the first phase of immunization the haemagglutination inhibiting factor is present at low concentration, at titres of 1 : 4—1 : 8 or it is entirely absent. Several months later, however, after large antigen or hyperimmunizing doses had been administered, the haemagglutination inhibiting titres ranged from 1 : 16 to 1 : 128.

The non-specific inhibitors were not inactivated at 56°C for 30 minutes, but could be removed from the sera by adsorption with kaolin.

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DISTRIBUTION OF SPECIFIC AND NON-SPECIFIC INHIBITORS AGAINST
VACCINIA VIRUS HAEMAGGLUTINATION IN PROTEIN FRACTIONS OF
IMMUNE SERA AND PATHOLOGIC BIOLOGICAL FLUIDS

Sera from rabbits and cows immunized against vaccinia virus were examined for specific haemagglutination inhibiting antibodies and pathologic biological fluids from man as well as hyperimmune antitoxic sera from horses for aspecific inhibitors, with respect to their distribution in the different protein fractions. The specific haemagglutination inhibiting antibodies were demonstrated in fractions I, II and III, containing large amounts of immune globulin. All aspecific inhibitors but one were found to have accumulated mainly in fraction I.

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FLUORESCENT TREPONEMA ANTIBODY (FTA) TESTS WITH ANTI-IgA,
ANTI-IgM AND ANTI-IgG CONJUGATES

Anti-human horse serum, anti-human IgA, IgG and IgM horse or rabbit sera were conjugated with fluorescein isothiocyanate. The fluorescein and protein contents of the individual conjugates were determined. The changes in the antibody level during infection and treatment were examined by indirect immunofluorescence, using the different conjugates.

In the sera of syphilitic subjects the two kinds of antibodies, viz. the antibody to the specific surface antigen of *Treponema pallidum* and that to the *Treponema* group-specific antigen were examined separately, using sera adsorbed with cultural treponemas. The FTA allowed to study the dynamism of the different antibodies (IgA, IgM, IgG).

The species- and group-specific IgA and IgM globulins appear early after infection. Antibodies of IgG type reach their maximum in the secondary phase. Group-specific antibodies are attached mainly to IgG globulins. IgA and IgM globulins contain group-specific antibodies only in smaller amounts.

From the diagnostic point of view, none of the conjugates seem to have any advantage over the other, each reacting with non-specific antibodies, too. Nevertheless, IgG being present in the greatest amounts and for the longest period of time, the anti-IgG conjugate appeared to be most useful for diagnostic purposes.

VIROLOGY

E. FARKAS, O. RUDNAI, É. CSONKA, S. LETENAY

*(National Institute of Public Health, Budapest)*A SEROLOGICAL AND EPIDEMIOLOGICAL SURVEY OF MEASLES
IN HUNGARY

On the basis of haemagglutination-inhibition (HI) test, antibody titrations carried out with 3298 serum samples collected from different areas of Hungary, about 3/4 of the six-years-old children population proved to be seropositive. For the school years the curve representing percentage positivity was less steep, over 20 years it declined slightly. This is because the HI antibody level of the serum may drop below the threshold of detectability several years after the measles attack. From the present data and the age distribution of the reported cases from 6 to 9 years of age the percentage of 10-year-old children who had experienced measles is estimated at 90 per cent. In contrast, only 22 per cent of the cohort 10 years old in 1963 had been reported with measles.

L. GÉDER, L. VÁCZI, F. LEHEL, E. JENEY, É. GÖNCZÖL

(Institute of Microbiology, University Medical School, Debrecen)"EARLY", "NON-VIRION" ANTIGENS IN CELLS OF TISSUE CULTURES
INFECTED WITH HERPES SIMPLEX VIRUS. I. DEMONSTRATION BY
COMPLEMENT FIXATION OF THE "EARLY" ANTIGENS

In BSC-1 permanent monkey kidney, human embryonic fibroblast and HeLa cell cultures, infected with 1–8 TCD₅₀/cell *Herpes simplex* virus, the appearance of virus specific, though non-viral "early" antigens was demonstrable after of 5.5 hours of incubation. Production of early antigen was not inhibited by the presence of 10 µg/ml of cytosine arabinoside, thus the substance cannot be considered a viral antigen.

The antigen was thermosensitive, ether sensitive, and exhibited a 50 per cent titre reduction on repeated freezing and thawing. The substance did not sediment on centrifugation at 100,000 r. p. m. for 1 hour. With the indirect immunofluorescence technique, the antigen was demonstrable in the nuclei; it was arranged mainly along the nuclear membrane.

Out of the 7 *Herpes simplex* rabbit immune sera tested, 3 reacted also with the "early" antigen. Nevertheless, no correlation was found between the titres against the early antigen and those giving positive complement fixation against viral antigen. None of the six human convalescent sera did react with the early component in 1 : 4 dilution.

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INDUCTION OF INTERFERON PRODUCTION BY HUMAN
ADENOVIRUSES

All human adenoviruses examined up to now (types 3, 4, 5, 6, 7, 8, 10, 12, 16, 27) have been found to induce the production of interferon in chick embryo fibroblast cultures. Treatment by heat or trypsin abolished the interferon inducing capacity of adenoviruses. Trypsinization being known to damage exclusively the B antigen of the virus, we supposed this antigen to be involved in the induction of interferon. Interferon obtained in these systems seems to have the same characters as any other interferon. Infection with adenovirus failed to induce interferon production in other cell types (mouse or hamster embryo fibroblast, L cells and BKH 21 cell strain).

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ROLE OF FREE FATTY ACIDS IN THE EARLY PHASE OF POLIOVIRUS
REPRODUCTION

The final virus yield was measured in suspended PMK III/1 permanent monkey kidney cells infected with type 1 (Mahoney) poliovirus in different media. It has been shown that in the presence of bovine albumin or free fatty acid (unsaturated potassium linoleate, saturated arachidic, palmitic or stearic acid, each at 10^{-5} M final concentration) the final virus yield was identical with that obtained in Parker No. 199 medium containing bovine albumin. In cells suspended in Hanks' solution the final yield was 10 times less. The presence of bovine albumin or free fatty acid in the Hanks' solution was required exclusively during the phase of penetration. Thus both factors appeared to increase the number of efficiently infected cells rather than the number of virions produced per cell.

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POLIOVIRUS RECEPTORS OF PMK III/1 PERMANENT MONKEY KIDNEY
AND HELA CELLS

The enhancement of virus yield by bovine albumin observable in monkey kidney cells has been examined in HeLa cells. The effect failed to appear in versenized HeLa cells, while it was clearly demonstrable in those trypsinized

for 5–20 minutes. The presence of bovine albumin reduced the lag phase by 30 minutes in both cell types as compared to the lag phase in plain Hanks' solution. Further trypsinization of HeLa cells prior to infection resulted in the disappearance of the effect. Trypsinization of monkey kidney cells even for 1 hour did not inhibit the bovine albumin to increase the final yield and to reduce the lag phase. It has been concluded that HeLa cells have three types of receptor, one on the blebs which interfere with efficient infection at low multiplicity. The other two types of receptor are identical in both the HeLa and monkey kidney cells, one of them being sensitive the other insensitive to the bovine albumin effect. The bovine albumin sensitive receptors of HeLa cells are destroyed by prolonged trypsin treatment.

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PENETRATION MECHANISM OF POLIOVIRUS
(WORKING HYPOTHESIS)¹

Two a priori suppositions are made. (1) The relative density of charges on the polioviruses' icosahedral virion are higher in the vertices than on the edges and facets. Thus, vertices are preferential sites in virus-cell interaction. (2) The receptor region of the cell comprises more or less regularly arranged "receptor sites" capable of reacting with the virion's vertices. The probability of hits during adsorption decreases in the following sequence. (a) vertex, edge or facet hits non-receptor regions or non-receptor sites; (b) one vertex hits one receptor site; (c) two vertices hit two receptor sites; (d) three vertices hit three receptor sites. Case (a) is not considered here, being regarded as not resulting in efficient attachment. Case (b) is considered transitory, ending up either in the release of virions or in cases identical with (c) and (d). In case (d), the resulting conformational changes in the receptor sites are sufficient to initiate the penetration process (pinocytosis). In case (c), penetration takes place only if the third vertex of the involved facet is attached to the non-receptor part of the area by hydrophobic bond through a fatty acid. Case (b) being infrequent, the absence of fatty acid results in a low incidence of efficient infection.

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THE ROLE OF AN ACTIVE PRINCIPLE OF HUMAN CELL EXTRACTS
IN THE INITIATION OF THE REPRODUCTIVE CYCLE OF CERTAIN
ENTEROVIRUSES

The naturally occurring virions of certain enterovirus types need the presence of human cell extracts for a satisfactory replication in monkey kidney cells. The multiplication promoting (MP) factor is present in cultures of human cell lines as well as in extracts of normal human organs and red blood cells. From many respects, the MP factor appeared to be identical with the haemagglutination inhibiting (HI) substance in the same extract. Within a certain limit (0.1 to 1.0 HI units), a direct relation was demonstrable between the dose and the rate of reproduction enhancement.

The MP factor is bound by the virions as well as by the monkey kidney cells. Nevertheless, its effect is not due to an improvement of adsorption. Satisfactory adsorption of virions to the monkey kidney cells was demonstrable also in the absence of the MP factor, nevertheless penetration occurred only in the latter's presence. The velocity of penetration of MP factor requiring virions was identical with that of the freely penetrating ones.

GY. TAKÁTSY

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ADSORPTION TO RED BLOOD CELL RECEPTORS OF THE IS AND
IR MUTANTS OF INFLUENZA A₂ STRAINS

In earlier studies a certain correlation has been demonstrated to exist in the adsorption to chick red blood cell receptors and inhibitors between the inhibitor-sensitive (IS) and inhibitor resistant (IR) mutants of influenza A₂ virus. The mutant IR was adsorbed to the receptor like the classical A strains, and was eluted enzymatically. Mutant IS, however, adsorbed to the receptors in a stable form and its elution was moderate. This rule was not valid for the IR and IS mutants selected from recently isolated A₂ strains. These mutants, independently of their affinity to the inhibitor, adsorbed readily to both untreated and formalized red blood cells, while they were eluted only moderately from native, and practically not at all, from formalized red blood cells. The degree of elution was not influenced by the pH, while at higher NaCl concentrations, 80–90 per cent of the adsorbed virus was eluted. The process was not enzymatic, but simply physical-chemical. Inhibitor present in normal horse serum did not neutralize the strains isolated in 1965, in contrast

to the A_2 strains isolated earlier. Nevertheless, the more recently isolated strains exhibited an increased sensitivity to the inhibitor. The findings indicate the continual variation of the A_2 virus surface.

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COMPARISON OF THE ANTIGENIC STRUCTURE OF IS AND
IR MUTANTS SELECTED FROM THE PROTOTYPE STRAIN ISOLATED
FROM INFLUENZA A_2 EPIDEMICS

The antigenic structure was compared by cross haemagglutination inhibition test of A_2 influenzavirus strains isolated in connection with five definite influenza epidemics experienced in Hungary in the period 1957–1965. The studies supported the earlier results suggesting the progressive antigen-transformation of A_2 virus. The differences in antigenic structure were equally demonstrable in both IS and IR mutants with the only difference that the characters were more conspicuous with IR mutants. By means of the sensitive immune serum adsorption test, no differences in antigenic structure could be demonstrated between the IR and IS mutants of one and the same strain.

K. BARB, GY. TAKÁTSY

(National Institute of Public Health, Budapest)

EXAMINATION OF DIFFERENT INFLUENZAVIRUS VACCINES IN
ANIMAL EXPERIMENT

The immunogenicity in guinea-pigs was examined of 9 (8 Hungarian and 1 foreign) subcutaneous influenza vaccines produced by different technologies. The immune response was registered by the haemagglutination inhibition test and complement fixation with V antigen. The "native" inactivator-free vaccine and two preparations inactivated with relatively low doses (2.5×10^5 and 5×10^5 rad.) of gamma rays induced an immune response closely identical to that caused by the standard formol vaccine. Immunogenicity of the foreign vaccine and of those inactivated with two higher doses (10^6 and 2×10^6 rad.) of gamma rays was below the standard. Vaccines adsorbed to aluminium hydroxide gel and prepared with an oil adjuvant were 30 and 200 times more potent than the standard as referred to their respective antigen contents.

E. MOLNÁR, T. KUBÁSZOVA

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RECENT DATA ON TICK-BORNE ENCEPHALITIS INCIDENCE IN
HUNGARY AS DERIVED FROM COMPARATIVE SEROLOGICAL STUDIES

Sera from 1037 patients with acute neurological diseases observed in the period 1963 to 1965 were examined with virus neutralization or haemagglutination inhibition test. Neutralizing antibodies to the Hungarian KEm₁ strain of tick-borne encephalitis virus were demonstrated in 23 and 10 per cent of encephalitis and aseptic meningitis cases, respectively. The overwhelming majority of serologically positive cases was detected among the persons taken ill during spring and autumn. Among 1007 sera, 718 were negative both in neutralization and haemagglutination inhibition test, while 129 sera were positive in both tests, and 165 sera gave a positive haemagglutination inhibition but a negative neutralization reaction. Based on these results it is supposed that beside tick-borne encephalitis virus also another arbovirus of Casals' group B is occurring in Hungary. The usual proportion of seropositive cases was 10 to 20 per cent, but in the area of the river Duna and its affluents it reached as much as 40 to 50 per cent.

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COMPARATIVE STUDY OF NEWCASTLE DISEASE VIRUS STRAINS
ISOLATED FROM BROILER CHICKENS

On a total 18 Newcastle disease virus (NDV) strains were examined for pathogenicity for 10-day old chick embryo, one-day old chick and 6-week-old susceptible chickens.

Minimum lethal doses for 10-day old chick embryo were 0.1×10^4 /ml— 0.1×10^8 /ml, with survival times from 54 to 194 hours. Fifty per cent lethal doses varied from 0.1×10^6 /ml to $0.1 \times 10^{9.4}$ /ml. The range of haemagglutination titres was 1 : 50—1 : 660.

The intracerebral pathogenicity indices (ICPI) determined in day-old chicks were 1.5—1.7 with 5 strains, 0.2—0.9 with 10 strains and 0.0 with 3 strains.

The intravenous pathogenicity indices (IVPI) were 1.8—2.5 with 6 strains, 0.1—0.6 with 12 strains.

The high haemagglutinating ability as well as the long average survival time of chick embryos indicate a low pathogenicity, yet there are exceptions.

Both ICPI and IVPI are suited alone also for the determination of the chick pathogenicity of a given strain. With one strain the two indices were dissimilar, indicating a dependence of pathogenicity on the route of infection.

M. SIMON

(Hungarian Army Medical Corps, Budapest)

COMPARISON OF THE HAEMAGGLUTININS OF ECHOVIRUS STRAINS OF IDENTICAL SEROTYPES

The haemagglutination of freshly isolated echovirus strains has been studied. Echovirus strains of identical serotype with haemagglutinating activity exhibited differences in the optimal conditions of haemagglutination (temperature, pH).

Each strain requires the presence of cations for optimum haemagglutinating activity. In isotonic solutions of different carbohydrates, haemagglutination was not observed until the addition of an adequate amount of cations. The stability of red blood cell-virion complexes was different for certain individual strains within one and the same serotype. Strains haemagglutinating exclusively at 4 °C exhibited elution at 37 °C.

From prototype strains of type 3, 11 and 12 echoviruses certain mutants were isolated containing non-infective haemagglutinin. The latter was supposed to represent "empty" capsides synthesized as a result of some failure in virion replication.

M. SIMON, P. RUZICKA

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A SIMPLE METHOD FOR ESTABLISHING THE HUMAN ORIGIN OF CELL STRAINS

Continuous maintenance of several cell lines in one and the same laboratory may result in contamination of one cell line by another. A regular checking of identity of a given cell line is, therefore, advisable.

A simple method has been developed to demonstrate contamination of animal cell lines with human cells. The method was based on our observation that haemagglutination by echoviruses was inhibited by cell extracts of human origin. When tested by this procedure, out of the seven cell lines obtained by one of us (RUZICKA, 1964) from monkey kidneys, three turned out to be human cells and one to be partially contaminated with cells of human origin. The same result was obtained by the usual serological tests. Thus, the method allows rapid screening of monkey cell lines for possible contamination with cells of human origin.

É. HORVÁTH

(Institute of Microbiology, University Medical School, Debrecen)

A NEW DEOXYURIDINE DERIVATIVE

The effect of 5-nitrodeoxyuridine, an antimetabolite synthesized by KLUEPFEL, MURTHY and SARTORI in 1964, on the synthesis of *Herpes simplex*, *Herpesvirus varicellae* and *Vaccinia* viruses was examined. For comparison, parallel experiments were done with two antimetabolites of known action, 5-iododeoxyuridine and 5-bromodeoxyuridine.

(1) In the first series of experiments, inhibition of the cytopathic effect of the above viruses by the antimetabolites at concentrations of 100, 10, 1 and 0.1 $\mu\text{g/ml}$ was studied. The compound 5-nitrodeoxyuridine (NIDU) had the strongest inhibitory action on *Herpesvirus varicellae*, causing an 80 per cent focus reduction even at a concentration of 1 $\mu\text{g/ml}$. At a concentration of 100 $\mu\text{g/ml}$, it reduced the CPD_{50} of *Herpes simplex* virus by 1.0–1.25 $\log_{10}$ units, whereas 5-iododeoxyuridine (IDU) and 5-bromodeoxyuridine (BUDr) at the same concentration reduced it by 3 $\log_{10}$ units. Plaque formation by *Vaccinia* virus was not influenced by 100 $\mu\text{g/ml}$ of NIDU, while it entirely ceased in the presence of 1 $\mu\text{g/ml}$ of IDU or BUDr.

(2) In the second series, the quantity of infective virus synthesized in the presence of the examined antimetabolites was assayed. In the presence of 1 $\mu\text{g/ml}$ of NIDU, no infective *Herpesvirus varicellae* was synthesized, whereas IDU and BUDr at the same concentrations did not inhibit its synthesis. Synthesis of infective *Herpes simplex* and *Vaccinia* virus took place in the presence of 100 $\mu\text{g/ml}$ of NIDU, although in much lower titres than in the control, the differences being 2.5 and 3 $\log_{10}$ units, respectively.

3. In the third series, the effect of NIDU, IDU and BUDr on the appearance of viral antigens was examined. All the three compounds had a distinct inhibitory effect on the formation of varicella antigen. While NIDU had no influence on the formation of *Herpesvirus* antigen, it was hardly demonstrable in the presence of 100 $\mu\text{g/ml}$ of the two other antimetabolites.

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(Institute of Microbiology, University Medical School, Szeged)

MYCOPLASMA CONTAMINATION OF DIFFERENT TISSUE CULTURES

In studies concerning interferon induction by different types of human adenoviruses grown in HeLa cells, it was necessary to examine the cells for a possible contamination with mycoplasmas. Simultaneously with the HeLa cell line, the other cell lines maintained in this laboratory (L and HEp-2 cell

lines and BHK-21 cell strain) were also checked. Culturing showed the HeLa and L cell lines to be contaminated, whereas cell line HEP-2 and cell strain BHK-21 were free of mycoplasma.

From the HeLa cell line the contamination was eliminated by hypotonic-kanamycin treatment as suggested by GORI and LEE. The application of aureomycin-containing medium (50 μ g/ml, HEYFLICK) similarly eliminated the mycoplasmas from the HeLa cells. With cell line L, however, the latter procedure failed, thus this mycoplasma was resistant to aureomycin. The resistance was demonstrable in serial passages of the cell line in the presence of aureomycin. This procedure appeared to facilitate the selection of aureomycin resistant mutants of mycoplasma.

MICROBIAL GENETICS AND VARIA

G. SZABÓ, S. VITÁLIS, I. BÉKÉSI

(Institute of Biology, University Medical School, Debrecen)

DIFFERENTIATION STUDIES ON MICROORGANISMS

Differentiation has been studied in *Streptomyces*. In this system, the process was more simple and readily controllable than in any metazoan organism. The system differs from the more primitive model, the sporulation of bacteria, in that it contains simultaneously interacting several hyphae exhibiting different trends of differentiation. This fact was considered important, permitting more general conclusions.

Based on the cytomorphological changes observed in a conidium-forming and a non-conidium-forming mutant of *Streptomyces griseus*, a method has been developed which allowed to estimate the importance of different differentiation factors by obtaining "yes" or "no" answers. A substance of polypeptide nature was isolated from the fermentation fluid of the conidium-forming mutant. The substance seemed to have a certain role in the regulation of differentiation of the homologous strain.

This hypothesis has been supported by the observation that the isolated substance combined with DNA and thus exerted an influence on the m-RNA and protein metabolism of the cells. A close relationship was demonstrable between the morphological changes and the events at the molecular level. In contrast to expectations, this substance involved in the process of differentiation appeared to have a de-repressor effect.

The possibility cannot be excluded that this or similar polypeptides or proteins may be involved in the regulation of differentiation of other organisms, too.

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PHYSIOLOGICAL, BIOCHEMICAL AND GENETIC STUDIES ON THE
PENICILLINASE PRODUCTION OF *E. COLI*

The penicillin resistance and the mechanism of action of penicillin on Gram-negative bacteria have been studied.

Penicillinase was isolated from *E. coli* rendered resistant to ampicillin. The physiology of enzyme production and the genetic aspects of the phenomenon were studied and it was stated that amp A locus as structural gene was always indispensable for the synthesis of a protein with penicillinase activity. If only the wild type allele of amp A locus is existing the amp A⁻, an inactive though chromatographically and immunologically identical protein, is produced. The amp B locus of the double mutant is not responsible for enzyme synthesis in the cell. Penicillinase activity is controlled by the amp A gene as structural gene, ensuring enhanced resistance together with the amp B gene. Gene amp A⁻ does not produce active enzyme and its inactivity is not influenced by gene amp B. All these observations were indicative of gene amp B being a regulatory gene.

A. DOBOZY, E. MARJAI

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REGULATION OF THE ALKALINE PHOSPHATASE OF *B. ANTHRACIS*

It has been shown by LANTOS and IVÁNOVICS that the production of alkaline phosphatase by *B. anthracis*, similarly to some other bacteria, is regulated by the presence of phosphate ions. We have found that in different purine requiring auxotrophs, the grade of repression is different as compared to the parental prototrophic strain. The back mutation from purine dependency to prototrophy, results in the return of repression to the original level. Thus, the purine synthesis regulating chromosomal segment must be related to the gene controlling the production of alkaline phosphatase. This genetic correlation could not, however, be tested directly in view of the scarcity of information concerning the possibilities of genetic recombination with *B. anthracis*.

E. MARJAI, A. DOBOZY, G. IVÁNOVICS

(*Institute of Microbiology, University Medical School, Szeged*)

B. ANTHRACIS AUXOTROPHS

The virulence of certain purine, pyrimidine and vitamin-dependent *B. anthracis* auxotrophs isolated in an earlier study has been examined.

The conventional virulence test showed all auxotrophs to have maintained their original virulence except for those specifically dependent on adenine.

These avirulent mutants were analysed biochemically. They were found to belong to two groups depending on the enzyme missing from the pathway of *de novo* purine synthesis. In one group, adenino succinase, whereas in the other adenino-succino synthetase was missing.

The loss of virulence parallel to the development of specific adenine auxotroph dependence may be interpreted in two ways. The lack of virulence is either the primary phenomenon or the result of unsatisfactory adenine content of the host's blood.

Groups of mice were infected intraperitoneally with 5 million germs of either pre-germinated virulent or avirulent adenine auxotroph anthrax bacilli. Simultaneously with the infection and also every two to four hours during the experiment, both groups received adenine intraperitoneally. At certain intervals some animals were killed and in their peritoneal cavity the number of colony formers were determined. With the virulent strain, an initial temporary decrease was followed by a gradual increase of the number of colony formers and bacteraemia soon developed. In contrast, the germ count of adenine auxotrophs exhibited a continuous decrease. When the same experiment was performed with capsulated bacteria in the exponential phase instead of spores, the rapid destruction and disappearance of adenine auxotrophs was conspicuous in the smears taken from the peritoneal cavity.

The apathogenicity of adenine auxotrophs was not caused by the absence of their toxin production. In cultures of the strain, toxin production was demonstrable by means of precipitation as well as by the typical cutaneous reaction after intracutaneous inoculation in guinea-pigs.

It has been concluded that adenine auxotrophs are poorly resistant to the protective mechanisms of the organism and their rapid destruction prevents the possibility of producing lethal amounts of toxin.

This supposition was further supported by the observation that in cultures containing fresh defibrinated mouse blood, the adenine-dependent mutants died rapidly even in the presence of adenine. Virulent mutants grew well in such media.

It seems, therefore, that the avirulence of adenine-dependent mutants is not due to the adenine supply of the organism. Exogenous adenine supply appears to permit full growth of *vitro*, but does not ensure the development of a cell structure identical with that of the prototrophic organisms. The cell structure of the former thus seems to be impaired both in *vitro* and in *vivo*. Structural impairment, while of no particular importance *in vitro*, is fatal for the microbe when it has to face the defensive mechanisms of the host.

I. KÉTYI, L. SZENDREY

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POSSIBILITIES OF A GENETIC, CONJUGATIONAL ANALYSIS WITH THE
E. COLI STRAIN 0101 : K/A/30, PATHOGENIC FOR SUCKLING MICE

Auxotrophic and antibiotic resistant mutants of strain *E. coli* 0101 : K/A/30, pathogenic for suckling mice, have been produced by UV irradiation, HNO_2 or hydroxylamine treatment.

In the conjugation system of *E. coli* K-12, our strain proved to be F^- . Out of the six markers examined (proline, threonine, lactose, methionine, histidine, streptomycin), the proline and threonine markers were transferred at low frequency from *E. coli* K-12 Hfr. The transfer of lactose region occurred only at low frequency and partly in an unstable form. Methionine, histidine and streptomycin markers failed to be transferred. Thus, strain 18A10 with the antigenic structure 0101 : K 30, proved to be fertile with *E. coli* K 12; there was nevertheless a considerable genetic inhomology between the two strains.

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INHIBITORY ACTION OF AMINO ACIDS ON THE REPRODUCTION OF
AN INDUCIBLE PHAGE

It has been demonstrated previously that in strain *E. coli* K-12 λ 28, mitomycin induction in the shift down state resulted in the release of prophage. The process was not inhibited by chloramphenicol and the primary activity of the released prophage persisted for long periods. On a minimal medium, the strain was sensitive to valine, while in its shift down state, to leucine. In the shift down state, phage production took place prior to the onset of cell multiplication. Valine and leucine inhibited both processes at similar concentrations and under similar experimental conditions. When induction was performed during the inhibition by amino acid and the medium was later supplemented with casein hydrolysate, phage production began after a shorter period than in systems in which induction was brought about after the release of inhibition. This would show that even during inhibition the vegetative cycle proceeds further than the mere release of prophage. In studies on the chloramphenicol effect, the probability arose that even a partial synthesis of the early proteins may take place. Further studies are required to elucidate the possibility of DNA replication. Synthesis of the late protein was definitely inhibited.

F. SZESZÁK, G. SZABÓ

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ANTIBIOTIC PRODUCTION OF HYPHAL FRACTIONS OF *STREPTOMYCES*
GRISEUS

To study the production of an antibiotic, the biosynthesis and its relation to the life cycle of the producing organism is important both theoretically and from the industrial point of view. The antibiotic producing activity of *Streptomyces* mycelium has been studied by several authors (SEVERINA and GORSKAYA, 1959; HOCKENHULL, 1960; MAJUMDAR and KUTZNER, 1962; NOMI, 1963).

The present experiments have been performed with a streptomycin producing strain of *Streptomyces griseus* (No. : 52—1). A method was developed for the measurement of the streptomycin producing activity of washed mycelia in short term experiments.

In our earlier studies we have shown that mycelia obtained from submerged cultures were heterogeneous, exhibiting several morphologically different hyphal types representing different phases of the life cycle. The mycelium was separated into several fractions by density gradient centrifugation after disintegration in Waring blender. Definite hyphal types were concentrated in certain fractions. It was supposed that different hyphae may differ in their streptomycin production. The streptomycin producing activity of hyphal fractions obtained from 45-hour old mycelium was examined and it was established that the streptomycin producing activity per mg total protein of different hyphal fractions varied considerably. The blended mycelium used as starting material produced more streptomycin than any of the fractions. Thus, during streptomycin production the different hyphal types of the mycelium seem to exert a synergistic effect.

Experiments were performed with different hyphal fractions of a 72-hour old mycelium in order to determine the streptomycin production in submerged cultures inoculated with these fractions. It was found that after 72-hour shaking more streptomycin was produced in cultures inoculated with certain hyphal fractions than in those inoculated with the blended starting material.

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EFFECT OF CYSTEINE ON THE DIVISION OF *ESCHERICHIA COLI*
MICROORGANISMS

The growth of *E. coli* 15T cultures and their colony-forming ability was inhibited by cysteine at a concentration of 1 mM.

RNA synthesis was instantly arrested when cysteine had been added to the cultures, while DNA synthesis stopped after a lapse of 30 min. The DNA content of the cells was then one and a half-fold of that of the untreated cells, while the RNA content remained unchanged.

Cysteine treatment resulted in an increase in the cold acid-soluble SH content of the cells.

Biochemically, an unbalanced metabolic state developed in which the DNA/RNA ratio shifted towards DNA.

It has been concluded that (1) the sensitivity to cysteine of DNA and RNA mechanisms may be different, and (2) addition of cysteine may alter the intracellular SH/SS ratio.

Both assumptions may account for the cell division inhibiting effect of cysteine.

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INHIBITION OF NUCLEIC ACID SYNTHESIS BY CYSTEINE IN ESCHERICHIA COLI

The effect of L-cysteine-HCl on the phosphorus metabolism of logarithmically growing *E. coli* B cells has been studied.

The distribution of incorporated ^{32}P activity in cysteine-treated cells exhibited an increase in the cold acid-soluble ^{32}P , while the ^{32}P activity of the nucleic acid fraction was found to decrease markedly. A detailed analysis of the cold acid-soluble fraction has shown an increase in the total cold acid-soluble phosphorus, inorganic phosphorus and the barium-soluble phosphorus contents. The synthesis of nucleic acids in general, and that of RNA in particular, was affected adversely by treatment with cysteine. The inhibition of synthetic process resulted in an increase of the cold acid-soluble *i. e.* "pool" phosphorus content.

Examination of the nature of accumulated phosphate esters gave no clue as to the possible mechanism of action of cysteine. In this connection we have to deal with enhanced phosphate esters which usually show an increase when cell division is inhibited.

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OLIGOSACCHARIDE UTILIZATION BY YEASTS

Saccharose, raffinose, maltose, lactose, trehalose, melibiose and cellobiose utilization of 12 yeast species (*Candida cloaceae*, *C. maltosa*, *Dekkero-*

ces dobzhanskii, *D. drosophylarum*, *D. lodderi*, *D. phaseolosporus*, *Hansenula beijerinckii*, *Saccharomyces carlsbergensis*, *S. kluyveri*, *S. pretoriensis*, *S. robertsii* and *Schwanniomyces alluvius*) has been studied in live cells, acetone treated cells, and cell-free extracts. All species but *C. maltosa* appeared to contain invertase. *C. cloaceae* contained instead of invertase a structure-bound endoglucosaccharase. *S. robertsii* contained endoglucosaccharase and some invertase. Four species contained endomaltase (*C. maltosa* and *S. carlsbergensis* structural, *S. pretoriensis* acetone resistant, *D. dobzhanskii* acetone-sensitive, according to earlier physiological characterization), whereas *S. robertsii* contained exomaltase. *S. carlsbergensis* and *S. kluyveri* exhibited strong and very weak melibiase activity, respectively. In the other five species assimilating maltose aerobically but not anaerobically, no constitutive maltase was demonstrable. In species with endomaltase or endosaccharase, a direct aerobic uptake without extracellular splitting of maltose and saccharose was demonstrated. None of the species took up any of the other examined oligosaccharides anaerobically. Trehalase and cellobiose were demonstrable in the species metabolizing the respective disaccharide, while none of the species had lactase. Invertases of different yeast species cleaved the components of a mixture of four transfer oligosaccharides produced by *Claviceps* invertase in different sequences.

L. KATONA, T. DEÁK, E. K. NOVÁK

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EFFECT OF INHIBITORS ON GLUCOSE UPTAKE BY *PROCANDIDA ALBICANS*

The effect of moniodoacetate (MIA), sodium fluoride (NaF), nystatine (Ny), uranyl nitrate (UN) and potassium sorbate (KS) had been examined on the glucose uptake of *Procandida* (*Candida*) *albicans*. Extracellular and intracellular glucose content was measured in a system containing 1 g washed cells, 0.1 g glucose and the respective inhibitor, in a total of 5 ml M/30 phosphate buffer pH 7.2. Considerable intracellular glucose accumulation was observed with 5×10^{-3} M MIA and 5×10^{-2} M NaF. Inhibition of the decrease of extracellular glucose concentration occurred only at higher inhibitor concentrations. Ny at concentrations of 10–40 $\mu\text{g}/\text{ml}$ had a similar effect. At higher concentrations it inhibited the decrease of extracellular glucose concentration, but caused only moderate or no accumulation. The latter effect was probably the result of an impairment of the cell membrane's steroids. This supposition seemed to be supported by the observation that even at lower concentrations, the initially increased glucose uptake tended to decrease on further incubation. UN and KS, at concentrations of 2.5×10^{-2} M, and 1000 $\mu\text{g}/\text{ml}$ or more, respectively, increasingly inhibited the fall of extracellular glu-

cose concentrations without, however, causing intracellular glucose accumulation. Since the effect of KS was very similar to that of UN, well-known as an uptake inhibitor, it was supposed that the primary fungistatic effect of KS is the inhibition of sugar uptake. It was shown in addition that a combination of UN or KS with any of the glycolysis inhibitors (MIA, NaF, Ny) caused an enhancement of intracellular glucose accumulation. Thus it was supposed that UN and KS, besides inhibiting glucose uptake, have also intracellular point of attack.

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SORBIC ACID INHIBITION IN YEASTS

The inhibitory effect of sorbic acid on the growth of 8 yeast species has been compared in Sabouraud glucose broth, using 300–600 and 500–1000 $\mu\text{g/ml}$ potassium sorbate for anaerobic and aerobic cultures, respectively. The cultures were incubated for three days. The sorbic acid sensitivity of different yeasts was different and depended on the conditions of incubation. Inhibition was more marked under anaerobic than under aerobic conditions. According to the extent of growth under aerobic conditions, the species examined could be classified into three groups.

(1) Temporary and partial inhibition by sorbic acid was observed with *Candida utilis*, *C. claussenii*, *C. pseudotropicalis* and particularly with *Procan-dida (Candida) tropicalis* and *Pc. albicans*. Determinations of the potassium sorbate in the performed parallel to the growth measurements showed that the partial inefficiency could be explained by the continuously decreasing concentration of the active substance. This decrease of concentration was found to be the results of oxidation of the substance by the yeasts. The species examined were capable of metabolizing potassium sorbate as sole carbon and energy source. Parallel to the metabolization of potassium sorbate, the rate of growth increased. Under the given experimental conditions, reduced glutathione increased the inhibitory effect of potassium sorbate by inhibiting its metabolization.

(2) The growth of *Saccharomyces cerevisiae* and *S. carlsbergensis* was permanently and considerably inhibited by sorbic acid. The inhibition was but moderately enhanced by reduced glutathione. These species failed to decompose or metabolize the inhibitor.

(3) The growth of *Candida krusei* was moderately inhibited by sorbic acid. Reduced glutathione failed to enhance inhibition. This species was unable to metabolize sorbic acid. It was, however, able to grow in the presence of an efficient concentration of the inhibitor and thus appeared to be relatively resistant.

L. FERENCZY, F. KEVEI

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MECHANISM OF ACTION OF ANTIFUNGAL STEROID GLYCOALKALOIDS

Two antifungal steroid glycoalkaloids of selective activity were isolated from *Solanum laciniatum*: solamargine (solasodine — glucose — 2 *M* rhamnose) and solasonine (solasodine — galactose — glucose — rhamnose). In addition to the compounds containing three sugar molecules, solasodine derivatives containing 5 and 4 or 2 and 1 sugar molecules were also available.

An antifungal effect was observed only with compounds containing 3 sugar molecules. These compounds produced molecule complexes with ergosterol. The complex-forming activity and antifungal action were related. A close correlation was demonstrable between the complex-forming activity and the release from the cells of potassium ions, amino acids, and peptides. The ergosterol containing cell membrane appeared to be the site of the primary action. The resistance of non-sensitive microorganisms is not the result of an enzymatic sugar decomposition, but of a lack of adsorption.

G. MESZES, J. KRAIOVANSZKY, E. CSEH, Z. BÖSZÖRMÉNYI

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ION AND AMINO ACID UPTAKE OF UNICELLULAR ALGAE

The cation (K^+ , Na^+), anion (Br^-) and glycine uptake by *Scenedesmus* (in some cases *Chlorella*) algae has been studied in order to elucidate some fundamental characters of the uptake procedure. Part of the experiments was conducted in synchronised cultures.

The time course of uptake started with a rapid exchange followed by a prolonged uptake absorption of a steady rate. Between these two phases there seemed to be a third, transitional phase reacting differently to light effects, as compared to the steady rate absorption.

The concentration curves of uptake could be classified into three groups. (1) Following saturation, the bromide uptake by *Scenedesmus* increased again at higher concentrations; (2) in the region examined, sodium and potassium uptake by *Scenedesmus* and bromide uptake by *Chlorella* followed a simple saturation curve; (3) the saturation type of curves was poorly demonstrable in the glycine uptake by *Scenedesmus*. The K_m values for the uptake by *Scenedesmus* exhibited the sequence, $K < Na < Br$.

Potassium and sodium ions mutually inhibit each other's uptake. Only part of the potassium absorbed is liable to exchange; an initial rapid phase could be detected also in the exchange.

As shown by some preliminary experiments with synchronized *Scenedesmus* cultures, the $V_{\max}$ of bromide uptake increases more or less steadily in the daytime phase of the cycle, parallel with the growth of cells.

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MEASUREMENT OF LYSIS OF NK/LY MOUSE TUMOUR AND
RED BLOOD CELLS BY A RADIOISOTOPE METHOD

Cells from the red blood cell free ascitic fluid of Swiss mice inoculated 6–10 days earlier with NK/lymphoma have been labelled with N_2 $^{51}CrO_4$. The efficiency of labelling was examined in relation to the time of labelling, the relative concentration of cells and label activity, and the age of cells. The labelling rate of cells increased parallel to the labelling activity, exhibiting a maximum at 50 million cells/ml saline and 30 minutes labelling time with 0.1 to 0.5 μC activity/ml. At 0 °C, the labelled tumour cells maintained their activity against red blood cells for several days.

The tumour cell lysing activity of homologous rabbit, chick and rat immune sera was examined by means of labelled tumour cells. Two washings following the labelling of cells were found to be satisfactory. The ^{51}Cr content of cells incubated for 90 minutes in a mixture of equal amounts of chick immune serum and guinea-pig complement did not appear in the fluid phase. A lytic effect was demonstrable only with rabbit and rat immune sera. Sera heated to 56°C for 30 minutes lost their lytic effect which could, however, be restored by the addition of fresh guinea-pig or rat serum. A definite amount (4–10 μl) of anti-tumour rat immune serum released a higher proportion of ^{51}Cr from identical numbers of red blood cells than from tumour cells.

L. EGLER, M. SCHMIDT

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BINDING TO 30 S RIBOSOMES OF DIFFERENT MEMBERS OF THE
STREPTOMYCIN FAMILY

The mechanism of binding of different antibiotics of the streptomycin family (streptomycin, dihydrostreptomycin, kanamycin, paromycin, neomycin) has been studied.

These antibiotics showed a certain regularity in cross resistance, viz. organisms resistant to neomycin were sensitive to all members of the family, and organisms resistant to kanamycin were resistant to streptomycin, but sensitive to neomycin. The streptomycin-resistant organism maintained its sen-

sitivity to both kanamycin and neomycin. This regularity was observed in both artificial mutants and several hundred fresh isolates. The few exceptions could be explained by the effect of streptomycin on permeability.

A decisive importance is attributed to the primary amino and imino groups in the capability to attach to 30S ribosomes. This was supported by the observation that deamination by nitrite abolished the substance's antibiotic activity.

It is supposed that in the resistant cells a mutation in the structure of the 30S component of the ribosomes took place which inhibited the binding of the antibiotic and in this way it does not occur that by the misreading of codes a disturbance in protein synthesis should result in the sensitive cells. This hypothesis seems to offer an explanation for the phenomena of sensitivity, resistance and dependence.

GY. NAGY

(Research Institute for Frozen Food Industry, Budapest)

DENATURATION OF BACTERIAL CYTOPLASMIC PROTEIN AT LOW TEMPERATURES

The micro-flora of pork meat comprises mainly organisms belonging to *Pseudomonas* and *Micrococcus* genera. The strains may be classified into four main groups according to their resistance to low temperatures: obligate psychrophiles, psychrotolerants, psychrotropic mesophiles, and obligate mesophile.

The cell suspensions in water containing 10^9 /ml of viable germs were rapidly frozen at -5 or -20°C and stored for 24 days at the same temperature. The cells were then disintegrated by ultrasonic treatment and the water soluble cytoplasmic proteins were isolated. Proteins were fractionated on Sephadex G 200 columns. Water soluble proteins of untreated control cells were similarly analysed to obtain reference values.

In strains with considerable resistance to low temperatures, the water soluble cytoplasmic protein desaggregated on exposure to -5 and -20°C . For *Micrococci* and *Pseudomonads*, temperatures of -5 and -20°C , respectively, had the greatest desaggregating effect. Desaggregation resulted in a prolongation of the lag phase.

In obligate mesophilic *Pseudomonas* strains, aggregation was considerable at -5°C and somewhat less at -20°C . The phenomenon was accompanied by cell death, resulting in the reduction of the 10^9 /ml viable germ count to 10^8 /ml at both temperatures, during 24 days of storage. Desaggregation of proteins proved to be a non-lethal, while their aggregation a lethal, denaturation.

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Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója

Műszaki szerkesztő: Farkas Sándor

A kézirat nyomdába érkezett: 1967. II. 8. — Terjedelem: 11,50 (A/5) ív, 16 ábra

67.63458 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

Die *Acta Microbiologica* veröffentlichen Abhandlungen aus dem Bereiche der Mikrobiologie in deutscher, englischer, französischer und russischer Sprache.

Die *Acta Microbiologica* erscheinen in Heften wechselnden Umfanges. Mehrere Hefte bilden einen Band.

Die zur Veröffentlichung bestimmten Manuskripte sind an folgende Adresse zu senden:

Acta Microbiologica, Budapest, Keleti Postahivatal, Postafiók 64.

An die gleiche Anschrift ist auch jede für die Redaktion bestimmte Korrespondenz zu richten.

Abonnementspreis pro Band: 165 Forint. Bestellbar bei dem Buch- und Zeitungs-Außenhandels-Unternehmen »Kultúra« (Budapest I., Fő utca 32. Bankkonto Nr. 43-790-057-181) oder bei seinen Auslandsvertretungen und Kommissionären.

Les *Acta Microbiologica* paraissent en français, allemand, anglais et russe et publient des travaux du domaine de la microbiologie.

Les *Acta Microbiologica* sont publiés sous forme de fascicules qui seront réunis en volumes.

On est prié d'envoyer les manuscrits destinés à la rédaction à l'adresse suivante:

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AKADÉMIAI KIADÓ, BUDAPEST

1967

ACTA MICROBIOL. ACAD. SCI. HUNG.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA
MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: BUDAPEST V., ALKOTMÁNY UTCA 21.

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PREFACE

The present volume of *Acta Microbiologica Academiae Scientiarum Hungaricae* has been published to commemorate the six hundredth anniversary of the foundation of the University of Pécs.

This volume embodies articles by *Professor K. Rauss* and his coworkers, Institute of Microbiology, University Medical School, Pécs.

We have the honour of expressing our sincere congratulations on this valuable collection and wish the institute further successful contributions in microbiology.

The Editorial Board

STUDIES ON THE VIRULENCE OF SHIGELLA FLEXNERI

By

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(Received August 1, 1966)

Summary. On the basis of the colonies' light refractivity, virulent and avirulent clones have been isolated from *Shigella flexneri* 2a and 3 strains. These cultures, some mutants of the WALTER REED team and MEL's streptomycin dependent attenuated strain were examined for growth characteristics, ability to cause keratoconjunctivitis in guinea pigs and chronic excretion in mice and infectivity for chick embryos inoculated intravenously and in the allantoic sac. The clones showed partly virulent, partly avirulent properties. Avirulent clones were divided into 3 groups: fully avirulent mutants, clones that had only lost their ability to penetrate, and the group represented by the hybrid strain of FORMAL *et al.* which had retained its penetrating capacity but was unable to multiply *in vivo*.

In a previous study [1] we have isolated virulent and avirulent clones from *Shigella flexneri* strains by selecting colonies with different refractivity. Mice were successfully infected with a few cells of our virulent clones. The finding has led to the elaboration of a suitable immunological model [2–4].

It has also been shown that 10-day-old chick embryos were highly sensitive to endotoxins [5]. The same degree of sensitivity was observed to virulent organisms and even to some avirulent strains administered intravenously [6].

Examination of mouse infectivity (number of cells necessary for bringing about a chronic excretion of shigellae in mice) and the chick embryo virulence test together with other methods allowed a grouping of avirulent mutants and offered thus a way for further studies on pathogenicity (virulence) factors. The present paper gives an account of these observations.

Materials and methods

Organisms. *Sh. flexneri* 2a and 3 strains were isolated freshly from routine material. Selected mutants and a *Sh. flexneri* 2a × *E. coli* hybrid of S. B. FORMAL and the streptomycin dependent vaccine strain of D. M. MEL were also examined.

Selection of mutants. The cultures were streaked onto agar plates and refractivity and structure of colonies were observed in oblique light as described by KERÉKES [7]. Colonies showing the required characters were subcultured several times in order to obtain constant forms or to ascertain the stability of the morphological mutant. Stable clones were maintained by freeze-drying.

Checking of antigenic structure was performed by agglutination in absorbed sera. In order to reveal concealed R-mutation, the cultures were tested for agglutination in 0.1 per cent acriflavine.

Growth was examined in broth. Sometimes the number of colony-producing units present in broth cultures was also determined. These experiments were performed on DAVIS's

minimal agar [8]. When on this medium the organism failed to multiply or grew poorly, it was cultured on the same medium supplemented with amino acids, purine-pyrimidine bases and different vitamins (B_1 , B_2 , B_6 , folic acid, PABA, nicotinamide, calcium pantothenate and biotin).

Growth in vivo was examined in the allantoic sac of 10-day-old chick embryos.

Antigenicity. Boivin extracts prepared from the clones were titrated by the haemagglutination inhibition method [9]. The intravenous chick embryo toxicity test [5] was also carried out. For active mouse protection test, mice were immunized subcutaneously then challenged 10 days later with intraperitoneal injections of 100 to 1000 LD₅₀ virulent culture suspended in mucin.

Mucin virulence test was carried out by injecting intraperitoneally 10-fold serial dilutions of cultures suspended in 5 per cent mucin. The number of surviving animals was recorded after 3 days observation.

Keratoconjunctivitis test. As described by SERÉNY [10], one loopful of culture was dropped in the lower conjunctival sac of the guinea pig. Observation lasted for 5 days.

Occasionally the test was performed more quantitatively, by means of a syringe with an intradermal cannula, one small drop of suspension containing a known number of bacteria was inoculated into the conjunctival sac.

Mouse infectivity. With rare exceptions mice which had been pre-treated with streptomycin were used. They were given through a metal tube oral doses of 5 to 10 mg streptomycin daily for 2 days, then infected in a similar manner with appropriate doses of the culture. Successful infection was indicated by the development of chronic excretion of *Shigella*. Five days and sometimes on subsequent occasions after infection the faeces of the animals were seeded on eosin methylene blue agar and *Shigella* colonies were checked serologically.

Intravenous chick embryo test. Dilutions of the culture were injected with a thin (No. 21) cannula into the veins of the chorioallantoic membrane after removal of the shell over the air sac, as described previously [5, 6]. Ten-day-old embryos were used; they were observed for 2 days after infection.

Intraallantoic chick embryo test. Dilutions of the culture were injected through the air sac into the allantoic sac. Observation lasted for 3 days.

Statistical analysis. The 50 per cent infective dose (ID₅₀) and lethal dose (LD₅₀) were estimated as described by KÄRBER [11].

Results

Examination of Sh. flexneri clones 2aV and 2aAV. In a *Sh. flexneri* 2a strain isolated freshly from routine material two kinds of colonial types were observed: one was characterized by highly refractive, compact, the other by slightly refractive, flat colonies. Both clones retained their properties in the subcultures. Of the highly refractive (wild-type?) mutant 10^2 to 10^3 cells produced severe keratoconjunctivitis in guinea pigs within 24 hours. Repeated experiments with the slightly refractive mutant showed that even in enormous doses it was unable to produce keratoconjunctivitis. On the basis of the keratoconjunctivitis test, which is a reliable indicator of virulence, the highly refractive clone was considered virulent (2aV), the slightly refractive avirulent (2aAV). No remarkable difference was shown in the antigenic structure of the two mutants; although culture 2aAV became agglutinable in group-specific serum 3, 4, indicating that no R-type mutation had occurred, it remained inagglutinable in acriflavine. Neither was there a difference in the antigenicity and toxicity of the two clones. As shown by the intravenous chick embryo test, the LD₅₀ for both cultures was $10^{6.75}$ cells. In the active mouse protection test with formalinized suspensions and Boivin extracts the two mutants were equivalent. The haemagglutination inhibition titre was 1:4800 for 2aV and

1 : 3200 for *2aAV*. The mucin virulence test revealed no difference; the LD_{50} was 5×10^0 for *2aV* and 5×10^1 for *2aAV*.

In addition to the keratoconjunctivitis method, some virulence tests revealed considerable difference between the two clones. Oral infection with a few cells of *2aV* of mice pre-treated with streptomycin resulted in a chronic excretion of the organism. In contrast, as much as 500 million cells of the avirulent (*2aAV*) culture were unable to produce infection, and the bacteria disappeared from the intestinal tract within some days. The intravenous chick embryo test gave similar results; less than 10 cells of the virulent clone caused fatal infection while the avirulent mutant's LD_{50} was 5000 cells, which approximated the toxic dose. If the bacteria were injected into the allantoic sac, the lethal dose of the virulent clone was 10^3 cells; under identical conditions the avirulent mutant in a dose as high as 5×10^8 cells did not kill the embryos.

Further experiments also confirmed the difference in virulence of the two clones. Although in high doses (10^5 cells), it was only the virulent clone which was able to produce infection in streptomycin-untreated mice. When the animals had been infected intraperitoneally and faecal excretion was examined, the avirulent culture was not excreted in the faeces, in contrast to the virulent clone which gave rise to a chronic carriership. A similar result was obtained with chicks, in which infection with the virulent clone ($ID_{50} = 50$ cells) resulted in a chronic excretion of the agent. Properties of clones *2aV* and *2aAV* are summarized in Table I.

Table I and the above data indicate that the loss of virulence in mutant *2aAV* was not due to R-mutation nor was the endotoxin content of the mutant less than that of its virulent counterpart. The striking inability of clone *2aAV* to multiply *in vivo* was especially well indicated by the intravenous chick embryo test, under the conditions of which there is no barrier to protect the embryos from infection.

It has been mentioned that on agar plates clone *2aAV* grew slower and in smaller colonies than clone *2aV*. The difference in growth rate, although it cannot be made responsible for the complete avirulence of the latter clone, might still be indicative of its origin.

A comparison of growth in broth revealed that although the generation time of the two clones was identical (about 22 minutes), the avirulent mutant showed a prolonged lag phase and a short logarithmic phase (Fig. 1). The difference in growth was more definite *in vivo*. When sublethal doses of the organisms were injected into the allantoic sac of 10-day-old chick embryos, the virulent clone showed a rapid logarithmic multiplication. In contrast, the growth curve of clone *2aAV* was flatter and even showed a decline, which pointed to an increased fragility of the mutant *in vivo*.

On minimal medium the difference in growth was even more marked. Clone *2aV* produced a good growth, while colonies of *2aAV* became visible

after 48 hours incubation only. Amino acids enhanced the rate of growth, but no marked dependence was revealed.

Examination of clones selected by FORMAL and MEL. It seemed interesting to apply our virulence tests to stable selected clones which had been character-

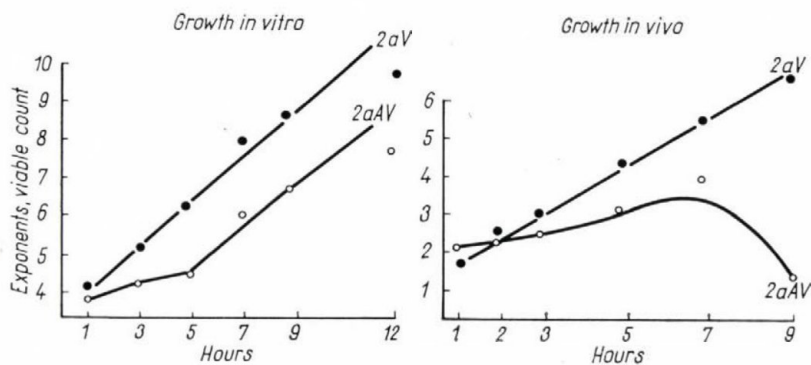


Fig. 1. Growth of *Sh. flexneri* 2a virulent and avirulent mutants *in vitro* and *in vivo*.

Table I

Properties of virulent and avirulent mutants selected from Sh. flexneri 2a on the basis of colonial refractivity

Properties	Virulent mutant 2aV	Avirulent mutant 2aAV
Refractivity	highly refractive	poorly refractive
Growth	normal	slow
Antigenic structure	(3, 4), II*	3, 4, II
Growth on minimal medium	+++	+
Growth on minimal medium + amino acid mixture	.	+++
Haemagglutination inhibition titre of Boivin extract	1 : 4800	1 : 3200
Immunogenicity	identical	
Toxicity	identical	
Virulence test, mucin technique	5×10^9 cells	5×10^1 cells
Keratoconjunctivitis test	+++	—
Infectivity (ID_{50}) to streptomycin-treated mice	5×10^9 cells	$> 5 \times 10^8$ cells
Infectivity to untreated mice	10^5 cells	$> 5 \times 10^8$ cells
Intraperitoneal dose causing shigella excretion in 50 per cent of mice	10^3 cells	$> 5 \times 10^8$ cells
Infectivity (ID_{50}) to streptomycin-treated chicks	$< 5 \times 10^1$ cells	$> 5 \times 10^8$ cells
Intravenous chick embryo test (LD_{50})	$< 10^1$ cells	$> 5 \times 10^3$ cells
Intraallantoic chick embryo test (LD_{50})	10^3 cells	$> 5 \times 10^8$ cells

* Inagglutinable in group serum 3, 4.

ized by other methods. The following clones were obtained from Dr. S. B. FORMAL:

(1) *Sh. flexneri* 2a strain 2457/0, an avirulent mutant selected by colonial light refraction. When tested for mouse virulence by the mucin technique, it was not different from the virulent parent strain (2457/T). In toxicity the two cultures were identical. Oral infection of guinea pigs, however, indicated a loss of virulence in the mutant which was unable to induce infection in monkeys after oral ingestion. On the basis of morphological examinations and growth experiments in HeLa cultures, the avirulence of the clone was attributed to a loss of the penetrating ability (LA BREC *et al.* [12]).

(2) *Sh. flexneri* 2a 2457/T—M22—18. The organism is a stable virulent subculture of a virulent clone of the parent strain, obtained by passaging in monkeys [12].

(3) *Sh. flexneri* 2a 2457/T $\times$ *E. coli* K12 W 1895 hybrid—M—22 18 $\times$ 16. The hybrid had acquired the rha-xyl region of *E. coli* K12. It has lost its pathogenicity for the guinea pig and the monkey, but retained its penetrating capacity. Multiplication *in vivo* of the hybrid is hindered and it is eliminated from the tissues of the animals in 4 days (FORMAL *et al.* [13]).

Streptomycin dependent strain *Sh. flexneri* 2a 2457TN SM^d, which had been used for human vaccination, was obtained from Dr. D. M. MEL. The streptomycin dependent clone had been isolated from strain 2457 T of the WALTER REED Army Institute of Research [14].

The above clones were tested by the keratoconjunctivitis, mouse infectivity and intravenous and intraallantoic chick embryo tests. In order to maintain the necessary streptomycin environment for MEL's streptomycin dependent strain, the guinea pigs were given in the conjunctival sac 400 μ g streptomycin twice daily and additional daily doses of 0.3 g streptomycin intraperitoneally. Mice and chick embryos were also provided with streptomycin to maintain a level of 100 to 400 μ g per g body weight. Results are summarized in Table II. As a comparison, data for clones 2aV and 2aAV are also presented.

As shown by our experiments, virulent clones M22—18 of the WALTER REED team was similar in virulence, even in the order of dose necessary for producing infection, to our clone 2aV. MEL's streptomycin dependent culture was similar to mutant 2aAV and its avirulent property was consequently due not only to its dependence, but also to its inability to multiply *in vivo*. The WALTER REED 2457/0 avirulent clone and the hybrid strain differed remarkably from the above cultures. Strain 2457/0 was avirulent when tested by the keratoconjunctivitis and intraallantoic chick embryo tests. The same held for the infection of mice, which was successful only when enormous doses (10^7 cells) were given. However, when injected intravenously into chick embryos the culture behaved like a virulent strain and was lethal in doses characteristic of virulent organisms. There was also a difference in the behaviour of the

Table II

*Biological properties of Formal's and Mel's
Sh. flexneri 2a clones and mutants 2aV and 2aAV*

Clones	Keratocon- junctivitis test	Mouse infectivity ID ₅₀ (cells)	Chick embryo test	
			intravenous LD ₅₀ (cells)	intraallantoic LD ₅₀ (cells)
FORMAL's M22-18				
virulent	+++	10 ¹	5 × 10 ⁰	10 ³
M22-18 × 16 hybrid	—	> 5 × 10 ⁷	10 ¹	2.5 × 10 ³
2457/0 avirulent	—	~10 ⁷	2.5 × 10 ¹	> 5 × 10 ⁸
MEL's 2457 TN S ^d	—	> 2 × 10 ⁸	5 × 10 ³	> 2 × 10 ⁸
2aV virulent	+++	5 × 10 ⁰	< 10 ¹	10 ³
2aAV avirulent	—	> 5 × 10 ⁸	7.5 × 10 ³	> 5 × 10 ⁸

hybrid strain, which was avirulent by the keratoconjunctivitis and mouse infectivity tests, but virulent for chick embryos.

Examination of Sh. flexneri 3 clones. One *Sh. flexneri* 3 culture isolated freshly from routine material yielded 7 clones differing in light refraction and colonial morphology. Four out of the 7 clones remained stable on subculturing, viz. 3 RV_I, highly refractive, compact colonies; 3 RV_h, refractive, large colonies with reticular structure; 3 RAV_d, poorly refractive colonies with concentric structure; 3 RAV_n, poorly refractive colonies.

Designations A and AV indicate the result of keratoconjunctivitis reaction: 3 RV_I and 3 RV_h caused a rapid positive reaction, 3 RAV_d was negative and 3 RAV_n produced a weak but purulent keratoconjunctivitis 5 days after infection.

There was some difference between the clones in antigenic structure, which, however, was independent from virulence: 3 RV_I, 6, 7, III; 3 RV_h, 6, 7, —; 3 RAV_d, 6, 7, III; 3 RAV_n, 6, —, III.

None of the clones agglutinated spontaneously in physiological saline or in 0.1 per cent acriflavine. As indicated by the haemagglutination inhibition and active mouse protection tests, the mutants and their Boivin extracts were identical in antigenicity and there was no difference between the clones in toxicity.

The result of the keratoconjunctivitis test has already been mentioned. The mutants did not differ in mouse virulence tested by the mucin technique. In contrast, there was a considerable difference in mouse infectivity; the two keratoconjunctivitis positive mutants (3 RV_I and RV_h) induced chronic excretion in mice in low doses (3 to 5 cells). Clone 3 RAV_d was unable to bring about

Table III

Properties of stable virulent and avirulent mutants selected from Sh. flexneri 3 on the basis of colonial refractivity

Properties	3 RV _I	3 RV _h	3 RAV _d	3 RAV _n
Colonial morphology	highly refractive	reticular	concentric	poorly refractive
Growth	normal	normal	slow	slow
Carbohydrate fermentation	—	late mal ⁺	—	sorbose ⁻
Growth on minimal medium	+++	+++	+	—
Growth on minimal medium + amino acid mixture	.	.	+++	.
Antigenic structure	6, 7, III	6, 7, —	6, 7, III	6, —, III
Haemagglutination inhibition titre of Boivin extract	identical			
Toxicity	identical			
Immunogenicity	identical			
Keratoconjunctivitis test	+++	+++	—	+ 5 days
Virulence test, mucin technique	5 × 10 ⁹ cells	10 ⁹ cells	2 × 10 ¹ cells	2 × 10 ¹ cells
Mouse infectivity (ID ₅₀)	3 × 10 ⁹ cells	5 × 10 ⁹ cells	> 5 × 10 ⁸ cells	2 × 10 ⁷ cells
Intravenous chick embryo test (LD ₅₀)	9 × 10 ⁹ cells	< 4 × 10 ¹ cells	5 × 10 ³ cells	3 × 10 ¹ cells
Intraallantoic chick embryo test (LD ₅₀)	10 ³ cells	10 ³ cells	> 5 × 10 ⁸ cells	> 5 × 10 ⁸ cells

carriership. Clone 3 RAV_n was infective in streptomycin-treated mice in high doses (2 × 10⁷ cells) only. By the intravenous chick embryo test, in addition to clones RV_I and RV_h, mutant 3 RAV_n was virulent, while 3 RAV_d was avirulent. In contrast, when introduced in the allantoic sac, only mutants 3 RV_I and 3 RV_h behaved like virulent bacteria; mutant 3 RAV_n, similarly to avirulent clone 3 RAV_d, caused no lethal reaction. The properties of the clones are summarized in Table III.

Grouping of avirulent mutants. Some of the examined clones showed positive reactions in all virulence tests (*Sh. flexneri* 2a clone 2aV and WALTER REED M22—18, *Sh. flexneri* 3 clones 3 RV_I and 3 RV_h). Mutants regarded avirulent were not uniform in the various virulence tests. The small number of avirulent mutants so far examined, may be divided into 3 groups (Table IV).

Table IV

Grouping of avirulent mutants on the basis of virulence tests

Virulence tests	Avirulent mutants		
	2aAV MEL'sTN S ^d	3 RAV _d 2457/0 3 RAV _n	M22—18×16 hybrid
Keratoconjunctivitis test	negative	negative or $\pm$	negative
Mouse infectivity	nil	weak	nil
Intravenous chick embryo test	approx. 10 ³	approx. 10 ¹	10 ¹
Intraallantoic chick embryo test	negative	negative	approx. 10 ³
Groups*	Completely avirulent, does not penetrate and multiply <i>in vivo</i>	Does not penetrate, but multiplies <i>in vivo</i>	Penetrates, but does not multiply <i>in vivo</i>

* See Discussion.

Sh. flexneri 2aAV, *Sh. flexneri* 3 RAV_d and MEL's mutant S^d behaved similarly. These clones were completely avirulent by keratoconjunctivitis, mouse infectivity and intraallantoic chick embryo tests. When injected intravenously into chick embryos, they were fatal only in doses of the order of 10³ cells. The second group was represented by *Sh. flexneri* 3 RAV_n and mutant 2457/0 of the WALTER REED team. These clones gave negative or very weak keratoconjunctivitis reaction, they infected mice in very high doses (approximately 10⁷ cells) only, were avirulent by the intraallantoic but virulent by the intravenous chick embryo test. Hybrid strain M22—18×16 behaved differently; it was avirulent for mice and caused no keratoconjunctivitis, but was virulent for chick embryos.

Discussion

On the basis of our present, very defective, knowledge, virulence seems to be of polygenic information. This consideration holds undoubtedly true for *Enterobacteriaceae* and thus for *Shigella*. The pathogenicity of these organisms is constituted of two factors; invasiveness and endotoxic effect. Invasiveness is a complex property *per se*; the causative agent penetrates the intestinal wall, then multiplies until the released endotoxin has reached or exceeded the toxic level. The ability to multiply *in vivo* obviously includes several different factors namely capacity to multiply, resistance to antibodies and lysozyme, etc.

Our conception agrees completely with that of the investigators of the WALTER REED group [12], according to whom the pathogenicity of shigella consists of the following phases. (1) Penetration into the lamina propria; (2) ability to multiply in the intestinal wall; (3) capacity to produce endotoxin.

Studies of this highly complex process may be greatly advanced by the investigation of mutants which had lost some of their virulence factors. R-mutants are well-known examples of such organisms. The loss of the surface lipopolysaccharide antigen is known to be associated with avirulence probably because this antigen is responsible for the antiphagocytic activity of the cell. The loss of this antigen, due mainly to the direct effect of R-mutation, is, however, accompanied by certain changes (less intensive growth, decreased resistance), which make it difficult to study the virulence. For such studies, S-form microorganisms seem therefore much more suitable.

In lack of a suitable selective technique isolation of clones with different virulence is not easy. The observation of COOPER in 1957 [15], that a *Sh. flexneri* culture consisted of colonies differing in refractivity when examined at oblique light, has been considered of great importance. KERÉKES [7] introduced a simple technique for showing the difference in refractivity and confirmed the connexion between colonial morphology and virulence to the guinea pig's eye.

The WALTER REED team as well as ourselves, used differences in light refractivity for the isolation of virulent and avirulent clones with intact antigenic structure. Methods for studying virulence, however, were different. First of all we determined the structure of surface antigens and endotoxin content of our virulent and avirulent clones. These examinations revealed that the observed differences in the keratoconjunctivitis reaction were not due to R-mutation or changes in endotoxin content.

Mouse infectivity tests yielded essentially the same results as the keratoconjunctivitis reaction. Since mouse infectivity is a complex process, this finding is obvious: failure of the organism either to penetrate or to multiply *in vivo* leads to a negative result. At present it is difficult to interpret the behaviour of avirulent clones 2457/0 and 3 RAV_n, which in high doses (in the order of 10^7) are both capable of inducing excretion. One explanation of this behaviour might be that these mutants represent a mixed population and in such large doses a reversion to virulence may occur. A further explanation is that, owing to the large dose, some cells may gain entrance "passively" into the lymphoid structures on the intestinal wall. In our opinion the latter explanation is more probable. For drawing final conclusions further studies are needed.

The intravenous and intraallantoic chick embryo tests allow some differentiation of the virulence factors. It may be supposed that, when administered intravenously, the organism needs no penetrating capacity to cause infection.

Thus mutants 2457/0 and 3 RAV_n, which showed virulence by the intravenous test but were avirulent when inoculated into the allantoic sac, had probably lost their ability to penetrate, but retained their power to multiply *in vivo* (Table IV). The chick embryo tests, therefore, can be used as indicators of penetrating capacity. For determining the ability to multiply *in vivo*, however, by itself the test seems inadequate; this is indicated by the behaviour of the hybrid strain, which obviously possesses the capacity to penetrate (positive intraallantoic test), but is unable to bring about keratoconjunctivitis and chronic excretion, owing probably to its limited multiplication *in vivo*. As it has been mentioned, growth *in vivo* is associated with a resistance to normal antibodies, enzymes, and other factors. Undoubtedly, the embryonated egg does not yet possess every possible antibacterial mechanism, that is, factors inhibiting the growth of the infecting organism are only partly present. This consideration is even more valid for the mouse virulence test performed with the mucin technique, since in this test virulent and avirulent strains reacted similarly. The three reactions (mouse virulence by the mucin technique, chick embryo test and mouse infectivity) are probably suitable for a more exact differentiation of the capacity to multiply *in vivo* and perhaps of the complex factors of survival.

The mechanism of penetration is entirely unknown; today it is impossible to say whether an enzymic or other mechanism is involved. According to the results of the WALTER REED team and our own observations, penetration seems to be an active property of the agent.

The experimental finding that our avirulent strains grew *in vitro* less readily than the virulent mutants, should also be considered. It is especially important that mutant 3 RAV_n, which had lost its capacity to penetrate, is a true auxotrophic mutant. These observations are in full agreement with STYPULKOWSKA-MISIUREWICZ's experimental data, who found that avirulent mutants of *Sh. flexneri* 2a and 3 are capable of utilizing a narrower range of various N-sources than do the virulent parent strains [16]. It is, of course, questionable whether dependence is associated with penetration or is an independent mutation.

Our observations are calling for more sensitive methods; first of all the partial factors influencing growth *in vivo* should be determined by some new procedure.

Studies of isolated impairment of partial virulence factors may open up a way for the determination of the biochemical basis of the different factors and thus for the explanation of the virulence mechanism in shigellae.

It is important to note that, by the use of entirely different methods, the WALTER REED researchers [12, 13] and our team have reached identical conclusions in many a theoretical problem of shigella pathomechanism.

Acknowledgements. We are indebted to *Dr. S. B. FORMAL* (Walter Reed Army Institute of Research, Washington, D. C.) and to *Dr. D. M. MEL* (Military Medical Academy, Belgrade) for supplying their cultures.

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IMMUNOGENICITY OF ORALLY ADMINISTERED AVIRULENT SHIGELLA CLONES

By

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(Received August 1, 1966)

Summary. The immunogenicity of attenuated *Shigella* strains with different characters has been examined. Living avirulent mutants which had lost their penetrating capacity were not superior to vaccines containing killed bacteria. The hybrid strain of the WALTER REED team, which had retained its penetrating capacity, was more effective in immunogenicity than other avirulent strains or killed organisms, but did not yield significantly better result than Boivin extracts. Thus, the safe and orally administrable Boivin antigen is at present the vaccine of choice. The aspects and limits of the application of attenuated *Shigella* strains are discussed.

Mouse experiments have shown that when a sufficient antigenic stimulus is used, oral immunization against shigella infection is equivalent with parenteral vaccination [1]. Further studies resulted in the elaboration of an experimental model named "mouse-shigellosis" consisting of oral streptomycin treatment and a subsequent oral infection of the mice with a few cells of a selected virulent strain [2]. This model has been found suitable for immunological studies [3, 4], as it yielded data for the ability of various vaccines and passive immunization to prevent the development of chronic carriership.

In previous experiments we obtained virulent and avirulent clones by selecting *Sh. flexneri* colonies with different refractivity [5]. In possession of our and other workers' avirulent mutants there was an opportunity to carry out experiments on the immunogenicity of cultures impaired in some of their virulence factors. This paper gives an account of these studies.

Materials and methods

Vaccines. Living vaccines were prepared from the following avirulent clones: "completely avirulent" mutants 2aAV and 3RAV_d isolated in this institute; completely avirulent and streptomycin dependent mutant 2457 TN S^d [5, 6] of MEL *et al.*; non-penetrating mutants 3RAV_n and 2457/0 (WALTER REED team) [5, 7]; hybrid strain M22—18×16 (WALTER REED team) [5, 8], which retained its penetrating power but exerted a limited multiplying capacity *in vivo*. All clones were S forms complete in antigenic structure.

Comparative examinations were carried out by use of vaccines killed with 0.3 per cent formalin, and Boivin extracts of virulent cultures 2aV and 3RV₁ of the homologous serotypes (*Sh. flexneri* 2a and 3).

Properties of virulent and avirulent clones have been described previously [5].

Oral immunization was performed with 5 doses given at 3 day intervals by use of a metal catheter. The animals were infected 5 days after the last immunizing dose.

Mouse-shigellosis model. The immunized and control mice were given 10 mg streptomycin orally on each of two subsequent days. On the third day they were infected with approx-

imately 5 virulent cells. The animals were kept in sterile glass cylinders placed in well-closing boxes and were fed during the observation period on sterile food containing 1 mg/g streptomycin. Five days after infection and sometimes also on later occasions, the faeces of the animals were streaked on eosin methylene blue agar. Excretion of shigellae was verified by agglutination test.

Standardization of antigens. Corpuscular vaccines were standardized by use of the Human N. I. H. substandard 10T preparation (Dr. I. Joó). The antigenic equivalence of bacterial vaccines and Boivin extracts was measured by the haemagglutination inhibition test [9].

Statistical analysis. The 50 per cent effective dose (ED_{50}) was determined by KÄRBER's method [10]. Testing of significance was performed by the χ^2 method.

Results

In the first step, the immunogenicity of living avirulent mutant 2aAV was compared with that of a killed vaccine and Boivin extract prepared from the same virulent organism (Table I).

It is seen in Table I that the ED_{50} value was represented by an approximately similar number of cells of living and formalinized vaccines. The Boivin antigen seemed to exert a better effect, although in protective value it did not differ significantly from the two other vaccines ($\chi^2 = 0.641$; $P \sim 0.5$).

Table I

*Immunogenicity of an avirulent clone of Sh.flexneri 2a,
of formalinized vaccine and of Boivin extract*

Vaccine	Cell count	Excreter/infected mice	ED_{50}
2aAV living	5×10^{10}	2/10	2×10^9
	10^{10}	3/10	
	2×10^9	4/10	
	4×10^8	6/10	
2aV formalinized	5×10^{10}	2/10	2.5×10^9
	10^{10}	3/9	
	2×10^9	4/9	
	4×10^8	5/9	
2aV Boivin extract	5×10^{10} *	0/10	6×10^8
	10^{10}	1/10	
	2×10^9	3/8	
	4×10^8	4/10	
	Control	8/10	

* Cell counts equivalent to the amount of Boivin antigen as standardized by haemagglutination inhibition test.

Table II

Immunogenicity of avirulent clones selected from Sh. flexneri 3 and formalinized vaccine

Vaccine	Cell count	Excreter/infected mice	ED ₅₀
3RV ₁ formalinized vaccine	5 × 10 ¹⁰	0/8	8 × 10 ⁸
	5 × 10 ⁹	1/7	
	5 × 10 ⁸	5/8	
3RAV _d living	5 × 10 ¹⁰	0/7	2 × 10 ⁸
	5 × 10 ⁹	2/7	
	5 × 10 ⁸	5/8	
3RAV _n living	5 × 10 ¹⁰	1/7	2.5 × 10 ⁸
	5 × 10 ⁹	2/7	
	5 × 10 ⁸	3/7	
	Control	7/9	

From a *Sh. flexneri* 3 strain two avirulent mutants were isolated: mutant 3RAV_d similarly to clone 2aAV, was completely avirulent, while mutant 3RAV_n lost only its penetrating power. Living vaccines prepared from these two mutants were compared with the formalinized suspension of the virulent clone (3RV₁). One of these experiments is presented in Table II.

The two avirulent mutants were not superior in immunogenicity to the formalinized vaccine.

In further experiments we compared the immunogenicity of avirulent mutants isolated and studied by other authors. The available cultures belonged to *Sh. flexneri* serotype 2a and accordingly the comparative experiments were carried out with our *Sh. flexneri* avirulent mutant 2aAV. Previous studies [5] showed that the streptomycin dependent mutant 2457 TN S^d of MEL *et al.* was similar to our mutant 2aAV in that both of them were unable to penetrate and multiply *in vivo*. Clone 2457/0 of the WALTER REED team had lost only its penetrating power. Hybrid strain M 22—18 × 16 retained its penetrating power but was impaired in its ability to multiply *in vivo*.

The immunogenicity of these four clones is compared in Table III.

There was no difference in the immunogenicity of completely avirulent mutants (2aAV and 2457 TN S^d) and non-penetrating mutant 2457/0. As follows from our earlier experiments these cultures did not immunize more effectively than killed vaccines. In contrast, of hybrid clone M22—18 × 16 about one tenth of the doses sufficed for inducing an identical level of immunity. The χ^2 test indicated a significant difference between the protectivity of this clone and the other three mutants; $\chi^2 = 6.302$ ($0.02 > P > 0.01$).

These data have been confirmed by the following experiments.

In the experiments presented in Table IV a formalized vaccine of virulent clone 2aV was compared with living vaccines prepared from avirulent clones 2aAV (completely avirulent) 2457/0 (non-penetrating) and M22—18×16 *E. coli* × *Sh. flexneri* 2a hybrid (penetrating but not multiplying *in vivo*). From the results it is evident that the completely avirulent (2aAV) and non-penetrating (2457/0) cells were equivalent in immunizing capacity with the formalinized vaccine, while the *E. coli* × *Sh. flexneri* recombinant, again yielded an immune response higher by approximately one exponent.

Thus the consideration that non-penetrating mutants do not immunize more effectively than killed vaccines, has been confirmed. Better results can be expected only from the use of avirulent strains which retained a penetrating, and a limited degree of multiplying, capacity, in other words, of organisms which are able to come into contact for a while with immunologically competent cells situated in the lymphoid tissue of the intestinal wall. In our experiments the hybrid strain of the WALTER REED team represented this type.

Table III

Immunogenicity of Sh. flexneri 2a clones of different origin and virulence

Living vaccine	Cell count	Excreter/infected mice	ED ₅₀
2aAV	5 × 10 ⁹	2/10	6 × 10 ⁷
	5 × 10 ⁸	3/9	
	5 × 10 ⁷	4/10	
	5 × 10 ⁶	7/10	
2457/0	5 × 10 ⁹	1/10	6 × 10 ⁷
	5 × 10 ⁸	3/9	
	5 × 10 ⁷	5/10	
	5 × 10 ⁶	7/10	
M22—18 × 16 hybrid	5 × 10 ⁹	0/9	8 × 10 ⁶
	5 × 10 ⁸	0/10	
	5 × 10 ⁷	3/9	
	5 × 10 ⁶	4/10	
2457 TN S ^d	5 × 10 ⁹	2/10	7 × 10 ⁷
	5 × 10 ⁸	2/9	
	5 × 10 ⁷	4/9	
	5 × 10 ⁶	7/9	
	Control	8/10	

Our studies on oral vaccination [1] have shown that, due probably to their being readily absorbed, colloidal antigens like Boivin extracts immunize much more effectively than bacterial vaccines. Our recent observations [11] have indicated that a significantly higher immunity is obtained on administering the oral vaccine simultaneously with streptomycin. In view of these findings it seemed desirable to compare the immunogenicity of living avirulent vaccines and Boivin extract given without and with streptomycin. One example of these experiments is presented in Table V.

The Boivin antigen was brought to equivalence with a formalinized standard suspension by use of the haemagglutination inhibition test. This titration showed that 5×10^8 cells corresponded to 35×10^{-4} ml Boivin extract. A parallel immunization was carried out with Boivin extract doses each containing 2 mg streptomycin. The mutant 2457 TN S^d of MEL *et al.*, which had been tested in epidemiological investigations, was used as a completely avirulent living vaccine. The other living vaccine employed in these experiments was prepared from hybrid strain M22—18 $\times$ 16.

ED₅₀ values presented in Table V indicated that the lowest degree of immunity was produced by strain 2457 TN S^d (completely avirulent). The Boivin extract was more effective although not significantly so ($\chi^2 = 1.302$;

Table IV

Immunogenicity of formalinized vaccine and of clones representing different categories of avirulent mutants

Vaccine	Cell count	Excreter/infected mice	ED ₅₀
Formalinized vaccine (2aV)	5×10^9	2/10	6×10^7
	5×10^8	3/10	
	5×10^7	4/10	
2aAV Completely avirulent	5×10^9	1/10	10^8
	5×10^8	4/10	
	5×10^7	5/10	
2457/0 Non-penetrating avirulent	5×10^9	2/10	8×10^7
	5×10^8	3/10	
	5×10^7	3/10	
M22—18 $\times$ 16 penetrating hybrid	5×10^9	0/10	7×10^6
	5×10^8	2/10	
	5×10^7	2/10	
	5×10^6	3/10	
	Control	8/10	

Table V

*Immunogenicity of hybrid M22-18×16, of strain S^d
of Mel et al. and of Boivin extract*

Vaccine	Cell count	Excreter/infected mice	ED ₅₀
M22-18×16 hybrid	5×10 ⁸	0/10	7×10 ⁵
	5×10 ⁷	1/10	
	5×10 ⁶	2/10	
	5×10 ⁵	5/10	
MEL's 2457 TN S ^d	5×10 ⁸	1/9	10 ⁷
	5×10 ⁷	3/9	
	5×10 ⁶	5/9	
Boivin	5×10 ⁸ *	0/10	6×10 ⁶
	5×10 ⁷	2/10	
	5×10 ⁶	4/10	
Boivin + 2 mg Sm**	5×10 ⁸ *	0/10	5×10 ⁵
	5×10 ⁷	0/10	
	5×10 ⁶	1/10	
	5×10 ⁵	3/10	
	Control	8/10	

* Cell counts equivalent to the amount of Boivin antigen as standardized by haemagglutination inhibition test.

** Boivin extract administered with streptomycin. See text.

0.3 > P > 0.2). The higher immunogenicity of Boivin extract given with streptomycin was significant even at the 2 per cent level ($\chi^2 = 5.611$; $P \sim 0.02$). The hybrid strain was somewhat more effective than the simple Boivin extract, but the difference was not significant ($\chi^2 = 1.176$; $P \sim 0.3$). On the other hand, as compared to Boivin extract given with streptomycin, the hybrid produced a somewhat lower (non-significant) degree of immunity ($\chi^2 = 1.568$; $P \sim 0.2$).

From repeated experiments it was evident that Boivin extracts induced a more effective immunity than killed or living avirulent vaccines. The immunogenicity of the vaccine prepared from the hybrid strain which retained its penetrating power was higher than that of non-penetrating strains, but did not significantly exceed the immunogenic value of Boivin extract.

Discussion

Attenuated bacterial and viral vaccines are widely used in human medicine. First of all these products should be safe in that no reversion to the virulent form should occur. The second important requirement consists of the vaccines being "attenuated" namely the vaccine strains should contain "certain and sufficient" partial factors of virulence. The term means primarily an intact antigenic structure and a limited degree of invasiveness and multiplying capacity. In other words, the agent should multiply only to a degree which is not harmful, but at the same time, in order to maintain a sufficient stimulus, it should persist long enough in the organism.

Artificial immunity against enteric bacteria has not yet been fully elucidated. Even typhoid vaccination, which has been used successfully since long is a debated problem. Much less is known about vaccination against dysentery. A search for adequate attenuated strains and a survey of their applicability for vaccination is considered important, since in case of good results a way opens for the elaboration of the most convenient oral vaccination.

ISTRATI [12] was the first to perform vaccination of man with apathogenic strains not giving rise to keratoconjunctivitis shigellosa. The virulence category of these strains is not known, but they protected some human volunteers against infection with virulent bacteria.

FORMAL *et al.* [13] performed experiments with hybrid strain M22—18×16, which has also been examined in the present studies. Their *Macaca mulatta* monkeys weighing 1.8 to 3.6 kg showed a marked immunity against a virulent strain after receiving one massive immunizing dose (5×10^{10}). Non-penetrating mutant 2457/0 induced an immunity equivalent with that elicited by the hybrid when given in 5 subsequent doses each containing 5×10^{10} cells. FORMAL *et al.* have noted that clone 2457/0 brought about an immunity similar to that induced by a killed vaccine.

MEL *et al.* [14] isolated a streptomycin dependent mutant from the WALTER REED team's virulent (wild-type) strain 2457/T. They carried out in a considerable number of volunteers an epidemiologically highly successful vaccination by a large amount of bacteria, a total of 19.1×10^{10} cells given in 5 doses. One eleventh of this dose was found to induce no immunity as far as there was no difference in the morbidity of the vaccinated and control groups.

Our findings were in complete agreement with those of the mentioned authors. Similarly to FORMAL *et al.* [13] we have also reached the logical conclusion that only those avirulent mutants which have retained their penetrating power and some of their invasiveness or ability to multiply *in vivo*, are capable of bringing about a more effective immunity than killed vaccines. In agreement with the findings of the mentioned authors, only hybrid strain

M22—18×16 was shown to possess such properties. It has been demonstrated that the hybrid is present in the intestinal wall for 4 days and causes a slight inflammation. No data are available, of course, as to whether the organism multiplied in the intestinal wall, or, which is more probable, merely surviving cells were recovered, indicated by the fact that high immunizing doses were needed.

In this regard it is still doubtful whether a strain with a limited multiplying capacity would yield better results. Although such bacteria afford a higher degree of protection, they might give rise to unrequired clinical and perhaps morphological changes. It is thus questionable whether the somewhat higher immunogenicity of the hybrid strain justifies the use of an attenuated vaccine. Even if the danger of side effects is neglected, the production, transport and preservation of such vaccines would evidently present difficulties [14]. In the light of this consideration the vaccine of choice is the Boivin extract which, in addition to its practically equivalent immunogenicity, is easy to prepare, concentrate and compress into tablets, and can be preserved almost indefinitely [15].

The streptomycin dependent strain of MEL *et al.* is a safe attenuated culture not only owing to its dependence but also because of another mutation which has rendered it a completely avirulent, non-penetrating organisms [5]. The immunizing potency of vaccines prepared from this strain was, however, not higher than that of killed vaccines. MEL *et al.* also observed that an excessively high number of cells were needed for the preparation of an effective polyvalent vaccine. Nevertheless, the principle advanced by these authors should not be disregarded. It would be advisable to examine the immunogenicity of streptomycin (or other drug) dependent clones possessing unaffected virulence factors. It is, of course, questionable whether there is a limit between highly effective immunogenicity and unrequired vaccination reactivity.

The present data allow to conclude that avirulent strains ensure better results than killed vaccines only if they had retained certain virulence factors. Among these factors especially the O antigen and the penetrating power should be mentioned. It is doubtful whether the reactivity to vaccines prepared from strains possessing all these factors can be decreased to a sufficient degree. In view of our present concept that O antigen polysaccharide chains are built over the lipid responsible for the endotoxic effect [16], the existence of endotoxinless mutants with intact antigenicity is improbable.

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MAINTENANCE OF ARTIFICIAL DYSENTERY IMMUNITY BY ORAL VACCINATION IN HUMANS

By

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(Received August 1, 1966)

Summary. Oral vaccine has been prepared with dialyzed Boivin extracts of *Sh. flexneri* 2a, 3 and *Sh. sonnei*. The antigens retained their immunogenicity after alcoholic precipitation and freeze drying and on oral administration had no toxic effect on humans.

Basic immunization consisted of 2 parenteral doses of adsorbed vaccine in one group and 1 booster dose in the other group immunized one year before. After an interval of one month both groups received weekly doses of oral vaccine over a period of 8 to 12 weeks.

As indicated by the passive mouse protection test performed with the mucin technique, oral revaccination maintained protective antibodies at high levels. In persons not treated with oral vaccine, the antibody titre fell within 3 months to low values of doubtful protective effect.

The effectiveness of oral revaccination was confirmed on the mouse-shigellosis model.

In the course of previous investigations, about 40,000 individuals were immunized with polyvalent adsorbed dysentery vaccine [4, 5]. As shown by epidemiological surveys, immunity persisted for 3 to 4 months [12].

In subsequent mouse experiments it has been demonstrated that oral antigenic stimuli in sufficient doses are equivalent with parenteral vaccination [1]. The equivalent parenteral:oral antigen ratio was about 1:100 for corpuscular vaccines and 1:30 for well absorbable colloidal (Boivin) antigen. For effective basic immunization, the colloidal antigen had to be given in oral doses 30 times higher than the parenteral dose.

It is known that small doses suffice for booster vaccination or the maintenance of immunity. The question thus arose whether the short-term immunity following parenteral vaccination with adsorbed antigen [12] could be prolonged with an oral preparation.

Meanwhile a mouse model test has been elaborated [8] which has been found suitable for examining the duration of immunity established with absorbed vaccine. From these experiments it has been concluded that an effective immunity rendering mice unsusceptible to shigella infection persists 28 to 35 days at most [2]. It has also been shown that weekly oral administration of a 5-fold amount of antigen induces prolonged immunity.

In view of the promising results of animal experiments, it seemed desirable to examine whether or not the above principles hold true for man, in other words, would small doses of oral vaccine be capable of maintaining the mouse-protecting antibody level induced by parenteral immunization.

Materials and methods

Adsorbed vaccine. Parenteral immunization was carried out with adsorbed typhoid-tetanus-dysentery vaccine. The vaccine was prepared by adsorbing the Boivin extract of the organisms to high dispersity $\text{Al}(\text{OH})_3$ gel [3]. Preparation, titration and checking of the antigen were performed as described previously [4, 5].

Preparation, titration and checking of oral vaccine. Boivin extracts were prepared from strains corresponding to the *Shigella* components of the adsorbed vaccine (*Sh. flexneri* 2a, *Sh. flexneri* 3 and *Sh. sonnei*). The dialyzed antigens were titrated by the haemagglutination inhibition test [6], then precipitated with 4 volumes of ethanol. After freeze drying the antigen was tableted with a suitable vehicle. Each tablet contained 0.5 ml of each Boivin extract equivalent to 50 haemagglutination-inhibiting units of the antigens. This amount was 5 times higher than the parenteral dose. Placebo tablets of similar appearance were prepared without antigen.

After alcoholic precipitation and tableting, the effect of the antigen was checked by the haemagglutination inhibition and active mouse protection test.

The tablets were examined for sterility and for oral toxicity to mice. Checking on about 20 human volunteers was performed also by giving one or more tablets.

Serum samples. The sera of immunized persons were mixed at equal portions. The pooled sera were stored at -20°C .

Active mouse protection test. Groups of mice were injected subcutaneously with graded doses of the antigen. After a 10 day interval the animals were infected intraperitoneally with the homologous culture suspended in 5 per cent mucin. The challenging doses represented 100 to 1000 LD_{50} .

Passive mouse protection test. Groups of mice were immunized subcutaneously with graded doses of serum pools (0.05—0.005—0.0005—0.00005 ml). On the next day the animals were challenged by intraperitoneal injection of cultures suspended in 5 per cent mucin (approximately 100 to 500 LD_{50}). The number of surviving animals was read after 3 days observation.

Passive protection test on mouse-shigellosis model. Mice used in these experiments were pretreated with streptomycin and kept under sterile conditions [8]. Each animal received daily oral doses of 0.5 ml serum diluted 1:5. The serum was given on two subsequent days before infection, on the day of infection and on two subsequent days after infection. Technical procedures and determination of the ratio of carrier mice were performed as described previously [9].

Statistical analysis. LD_{50} and ED_{50} values were estimated as described by KÄRBER [7]. Significance was determined at the 5 per cent level by the χ^2 test.

Results

Preparation and checking of antigens for oral immunization. As indicated by animal experiments, the persistence of immunity can be prolonged by weekly oral immunization with 5-fold amounts of the subcutaneous dose of the adsorbed antigen. As the parenteral dose (10 haemagglutination-inhibiting units) was represented by 0.1 ml Boivin extract for each component (*Sh. flexneri* 2a, 3 and *Sh. sonnei*) in one oral dose 0.5 ml extract of each of the three components, thus a total of 1.5 ml antigen was incorporated. Tableting of antigens seemed the best method. This ensured an easy administration of exact doses and, by use of placebo tablets, an adequate control.

Since the use of dry substances is requisite for tableting, it is essential that the antigens keep their immunogenicity after drying. Moreover, the procedure should be suitable for large scale production. In order to meet these requirements, the Boivin extracts were dialyzed against tap and distilled water, then precipitated with 4 volumes of ethanol. The precipitate was mixed

Table I

Immunogenicity of Sh. flexneri 2a Boivin extract precipitated with 4 volumes of ethanol as compared to the standard extract

	Original extract	Dried alcoholic precipitate
ED ₅₀ (ml)	0.0001	0.00008
χ^2 test	0.164 (P ~ 0.7)	
Challenging dose (<i>Sh. flexneri</i> 2a)	100 LD ₅₀	

with a small volume of water and freeze dried. Freeze drying immediately after dialysis and precipitation was necessary to ensure a ready solubility of the preparation.

Alcoholic precipitation exerted no influence on the antigenicity of the extract. The precipitated and dried antigen retained its original haemagglutination inhibition titre and, as shown in Table I, its immunogenicity for mice remained unaltered.

Toxicity for mice of the dried antigen was also examined. The maximum solubility of the preparation was 50 mg/ml. Oral administration of 50, 10, 2 and 0.4 mg amounts exerted no toxic effect.

The preparation proved sterile in the usual tests.

The tablets were prepared with a suitable vehicle without preservative. One tablet contained 3 freeze dried antigenic components each corresponding to 0.5 ml Boivin extract. Solubility of the tabletted antigens was good; the supernatant over the deposit formed by the vehicle contained almost the full amount of incorporated antigen. Antigen titration was performed with haemagglutination inhibition and active (mucin technique) mouse protection test. Haemagglutination inhibition titres were identical with titres obtained with the original antigen. Results of mouse protection test are summarized in Table II.

It is evident that the tabletted and standard antigens did not differ in immunogenicity.

Finally the tablets were checked in human volunteers. Each of 20 persons received one tablet at 4 to 5 day intervals. Each of three persons were given 5 tablets in one dose. The tablets caused no appreciable symptoms in any of the volunteers. No harmful effect was observed in persons given 5 tablets in one dose, although one of them had recovered from dysentery caused by *Sh. flexneri* 3 one or two weeks before vaccination.

Vaccination schedule. Immunological examination of the combined parenteral—oral vaccine was performed on Army personnel. Originally 3 groups

Table II

*Immunogenicity of dried and tabletted Shigella antigens
as compared to standard extracts*

	ED ₅₀ values (ml)		
	Sh. flexneri 2a	Sh. flexneri 3	Sh. sonnei
Standard Boivin antigen	0.0004	0.0016	0.0025
Tabletted antigen	0.0006	0.0010	0.0010
χ^2 test	0.240	0.013	0.960
	0.7 > P > 0.5	P ~ 0.9	0.5 > P > 0.3
Challenging dose	250 LD ₅₀	500 LD ₅₀	1000 LD ₅₀

Table III

The realized immunization schedule

Weeks	Group A		Group B	
	Basic dose group	Booster dose group	Basic dose group	Booster dose group
0	Blood sampling Adsorbed vaccine	Blood sampling —	Blood sampling Adsorbed vaccine	Blood sampling Adsorbed vaccine
4	Adsorbed vaccine	Adsorbed vaccine	Adsorbed vaccine	—
7	—	—	Blood sampling	Blood sampling
8	Blood sampling	Blood sampling	—	—
<i>Both subgroups</i>				
9	One tablet	Control:	One tablet	Control:
10	weekly for	placebo	weekly for	placebo
11	8 weeks	tablets	12 weeks	tablets
12				
13				
14				
15				
16				
17	Blood sampling	Blood sampling		
18				
19				
20				
23			Blood sampling	Blood sampling

were examined. The results were evaluated in 2 groups (A and B). Both groups were divided into 2 subgroups. Members of subgroup I received dysentery vaccination for the first time and were given 2 doses of typhoid-dysentery-tetanus adsorbed vaccine at intervals of 4 to 5 weeks ("basic dose group"). Members of subgroup II had received 2 doses of combined dysentery vaccine one year before the examinations; they were therefore reinjected with only one typhoid-dysentery-tetanus dose ("booster dose group"). In group A oral revaccination with a total of 8 tablets given at weekly intervals, was commenced 1 month after parenteral immunization. Members of group B were revaccinated in a similar manner, but the weekly dose of one tablet was administered over a period of 12 weeks. In the last group the parenteral booster dose for subgroup II was given simultaneously with the first dose for subgroup I; consequently, in subgroup II oral revaccination was commenced 7 weeks after parenteral immunization. Subgroups I and II in both groups (A and B) were further divided into persons receiving antigen tablets and into persons receiving placebo tablets.

Blood samples were taken before parenteral vaccination, then 3 to 4 (in subgroup II of group B, 7) weeks after parenteral vaccination, that is before starting the administration of tablets; subsequent sampling was performed 1 (group A) and 3 (group B) weeks after oral vaccination had been completed. In the control group these periods corresponded to 13 weeks (group A), 19 weeks (group B, basic dose subgroup), and 23 weeks (group B, booster dose subgroup) after parenteral immunization. The realized immunization schedule is presented in Table III.

Immunological examination of serum pools

(1) *Passive mouse protection test with the mucin technique.* As mentioned under "Materials and methods" individual serum samples were mixed at equal portions according to the examined groups. Mouse protective antibodies were titrated in one experiment for all serum pools. Table IV demonstrates the protective antibody response for component *Sh. flexneri* 2a.

Parenteral injection of adsorbed typhoid-dysentery-tetanus vaccine induced after 3 to 4 (in B-II group 7) weeks a considerable (31 to 177-fold) rise in protective titre. In the non-revaccinated placebo group the titre decreased in 13 weeks to 10 to 17-fold and in 19 to 23 weeks to 5 to 10-fold of the titre observed before immunization. In persons vaccinated orally for either 8 or 12 weeks, the antibody titres showed no decrease 1 week (group A) and 3 weeks (group B) after completing the course. The titres in these groups remained at the maximum level observed 3 to 7 weeks after parenteral vaccination (55 to 178-fold of the original titre).

Similar results were obtained for component *Sh. flexneri* 3 (Table V).

As demonstrated in Table VI, *Sh. sonnei* yielded essentially the same results.

From the passive mouse protection tests it may be concluded that 2 injections or 1 booster dose of adsorbed vaccine produce high titre protective antibodies persisting for at least 2 months. After 4 to 5 months the titres decrease considerably and their protective effect becomes doubtful. Oral revaccination with small antigen doses results in the persistence of antibodies at a maximum titre. It is justified therefore to conclude that oral vaccination effectively prolongs immunity (Table VII).

Passive mouse protection test using the mouse-shigellosis model. The protective effect of some representative serum pools were examined on the mouse-shigellosis model. Immunization was performed orally. These experi-

Table IV

Mouse-protective antibody titres of serum pools against Sh. flexneri 2a infection
(Challenging dose, 250 LD₅₀)

Time after vaccination	ED ₅₀ values and (RP)							
	Group A				Group B			
	Basic dose group		Booster dose group		Basic dose group		Booster dose group	
	Pro- longed	Placebo	Pro- longed	Placebo	Pro- longed	Placebo	Pro- longed	Placebo
Before vaccination	~0.05* (1)		0.016* (1)		> 0.05* (1)		~0.05* (1)	
3 weeks after parenteral vaccination	.		.		0.0016* (> 31)		.	
4 weeks after parenteral vaccination	0.0005 (100)	0.0009 (55)	0.0001 (160)	0.0009 (177)	.		.	
7 weeks after parenteral vaccination							0.0009* (55)	
13 weeks after parenteral vaccination, 1 week after administration of 8 tablets	0.0003 (166)	0.005 (10)	0.0003 (53)	0.009 (17)	.		.	
19 weeks after parenteral vaccination,** 3 weeks after administration of 12 tablets					0.0009 (55)	0.009 (> 5)	0.0005 (100)	0.005 (10)

* Prolonged and placebo groups examined together

** In booster dose group 23 weeks after administration of adsorbed vaccine

ments revealed an extraordinary degree of passive immunity inhibiting the development of shigella carriership in mice. Results are shown in Table VIII.

Table VIII demonstrates results coincident with those of the passive mouse protection test performed with the mucin technique. Sera taken after parenteral vaccination inhibited shigella excretion significantly, the serum exerted an antiinfective effect. This immunity of parenterally vaccinated persons decreased after 3 months to such an extent that the ratio excreter/infected mice was about the same as that obtained with normal sera taken before vaccination. Oral vaccination effectively prolonged the duration of antiinfective immunity. The sera of this group of persons exhibited the same antiinfective effect as sera taken 3 weeks after parenteral immunization.

Table V

Mouse-protective antibody titres of serum pools against Sh.flexneri 3 infection
(Challenging dose, 100 LD₅₀)

Time after vaccination	ED ₅₀ values and (RP)							
	Group A				Group B			
	Basic dose group		Booster dose group		Basic dose group		Booster dose group	
	Pro-longed	Placebo	Pro-longed	Placebo	Pro-longed	Placebo	Pro-longed	Placebo
Before vaccination	> 0.05* (1)		> 0.05* (1)		~ 0.05* (1)		> 0.05* (1)	
3 weeks after parenteral vaccination	.		.		0.0016* (> 31)		.	
4 weeks after parenteral vaccination	0.0016 (> 31)	0.0009 (> 55)	0.0009 (> 55)	0.0009 (> 55)	.		.	
7 weeks after parenteral vaccination	.		.		.		0.0016* (> 31)	
13 weeks after parenteral vaccination, 1 week after administration of 8 tablets	0.0009 (> 55)	0.009 (> 5)	0.0005 (> 100)	0.009 (> 5)	.		.	
19 weeks after parenteral vaccination,** 3 weeks after administration of 12 tablets					0.0016 (31)	0.016 (3)	0.003 (> 17)	0.009 (> 5)

* Prolonged and placebo groups examined together.

* In booster dose group 23 weeks after administration of adsorbed vaccine.

Table VI

Mouse-protective antibody titres of serum pools against Sh. sonnei infection
(Challenging dose, 500 Ld₅₀)

Time after vaccination	ED ₅₀ values and (RP)							
	Group A				Group B			
	Basic dose group		Booster dose group		Basic dose group		Booster dose group	
	Pro-longed	Placebo	Pro-longed	Placebo	Pro-longed	Placebo	Pro-longed	Placebo
Before vaccination	0.05* (1)		> 0.05* (1)		0.05* (1)		> 0.05* (1)	
3 weeks after parenteral vaccination	.		.		0.0016* (31)		.	
4 weeks after parenteral vaccination	0.0016 (31)	0.0005 (100)	0.0005 (> 100)	0.0009 (> 55)	.		.	
7 weeks after parenteral vaccination	.		.		.		0.0009* (55)	
13 weeks after parenteral vaccination, 1 week after administration of 8 tablets	0.0009 (55)	0.005 (10)	0.0003 (> 166)	0.005 (> 10)	.		.	
19 weeks after parenteral vaccination,** 3 weeks after administration of 12 tablets	.	.	.	.	0.003 (17)	0.016 (3)	0.0016 (31)	0.016 (3)

* Prolonged and placebo groups examined together.

** In booster dose group 23 weeks after administration of adsorbed vaccine.

Table VII

Persistence and prolongation of artificial dysentery immunity, summarized on the basis of mouse-protective antibody titres

Time after vaccination with adsorbed antigen	Rise in protective value as compared to original titres
3—4 weeks	Considerable rise (31—177-fold)
2 months	No decrease (31—55-fold)
4—5 months	Considerable decrease (3—10-fold), protective effect doubtful
4—5 months with oral prolongation	Maximum level titres (31—66-fold)

Table VIII

Protective effect of serum pools on oral administration to mice
 Mouse shigellosis model; infecting dose: *Sh.flexneri* 2aV, 5 cells

Time after vaccination	Excreter/infected mice	χ^2 (compared to prevaccination result)
Before vaccination	4/10	1.818
3 weeks after parenteral vaccination	2/10	5.050
3 months after parenteral vaccination	4/10	1.818
4 months after parenteral vaccination, 3 weeks after administration of 12 tablets	1/10	7.500
Non-immunized control	7/10	.

□ = Significant difference from control.

Discussion

In artificial immunity against bacillary dysentery we are confronted with two difficult problems. First, owing to the multiplicity of the causative agent, effective immunity can be attained only with large antigen doses producing severe local and general reactions. Second, as immunity lasts for short periods, revaccinations should be repeated frequently.

In lack of a chemical method, toxicity of the vaccines can be decreased in two ways. One of them is the use of a suitable adsorbent, which if applied in suitable amounts, enhances the antigenic stimulus but decreases the toxic effect of the antigen ("functional detoxication"). This functional detoxication has been confirmed in animal experiments: mice tolerated the adsorbed preparation in amounts 8 to 10 times higher than the non-adsorbed antigen and at the same time the antigenicity of the adsorbate was enhanced about 10-times [4]. The adsorbed vaccine caused practically no reaction in man [5].

The other method for decreasing toxicity is oral administration, which allows the application of very high doses without any danger of a local or general reaction. The only disadvantage is that large amounts of antigen are to be prepared. Animal experiments have shown that, in order to produce a protective antibody response equivalent to parenteral immunity, corpuscular antigens should be used in 100-fold, Boivin extracts at 30-fold doses [1]. In spite of the difficulties, immunization with oral antigens has extensively been investigated since 1923. The studies have been reviewed by RAETIG [10] who advocated oral immunization himself.

In view of the short duration of immunity to dysentery and, consequently the necessity for frequent and early revaccination, the advantages of

the oral route are obvious. Thus, although basic immunity is established parenterally, oral revaccination is not merely a simpler procedure, but perhaps the only practical method.

This conception has been confirmed by animal experiments showing that an effective prolongation of immunity can be achieved with small antigen doses given at weekly intervals [2]. FRETER and GANGAROSA observed similar results for immunization against cholera [11].

The present experiments indicate that Boivin antigens can be dried and tabletted without a loss of immunogenicity. This antigen can be administered without danger; as noted by RAETTIG [10], the use of oral vaccines has practically no contraindication.

In earlier experiments the effect of oral vaccination was studied chiefly by titrating serum agglutinins. We have studied both the mouse-protecting and the antiinfective potency of the sera. Oral revaccination maintained the protective antibodies at high levels during the 19–23 weeks course of the experiment, as indicated by the passive mouse protection test performed with the mucin technique. In persons not treated with oral vaccine the protective titre fell to the starting point within 3 months.

We attached great importance to the results obtained in mouse shigellosis experiments. Our observations in mouse shigellosis, a condition analogous in pathomechanism with human dysentery, indicate namely that shigella immunity is based on humoral antiinfective antibodies, which act in the intestinal tract as “coproantibodies” [2, 8, 9].

As indicated by mouse shigellosis experiments, sera of the immunized persons have a marked antiinfective potency. Accordingly, there is no reason to be sceptical about the effectiveness of protective antibodies produced on either parenteral or oral immunization. Our experiments have confirmed that 4 to 5 months after parenteral vaccination the protective antibody titre is not high enough to ensure an effective immunity.

Although not closely connected with the subject of the present paper, it seems interesting to compare the immune response of persons who had their first vaccination one year before the experiments had commenced (“booster dose group”) and of persons not immunized previously (“basic dose group”). According to Tables IV, V and VI the antibody titres in the two groups showed no significant differences. This observation indicates that revaccination in the former group induced no booster effect. As confirmed by epidemiological findings [12] immunity disappears after a certain period; accordingly, the immunological state of the “booster dose group” was identical with that of the “basic dose group”. It follows from this consideration that a single dose of the adsorbed vaccine had induced a maximum immunity which could not be increased with a second dose. Data for persons revaccinated orally have confirmed this conclusion.

The effectiveness of the described dysentery vaccination method parenteral (basic immunization and oral revaccination) should be confirmed in extensive epidemiological investigations. Further studies are needed to throw light on the applicability of oral basic immunization, best interval of revaccination, and optimum immunizing doses.

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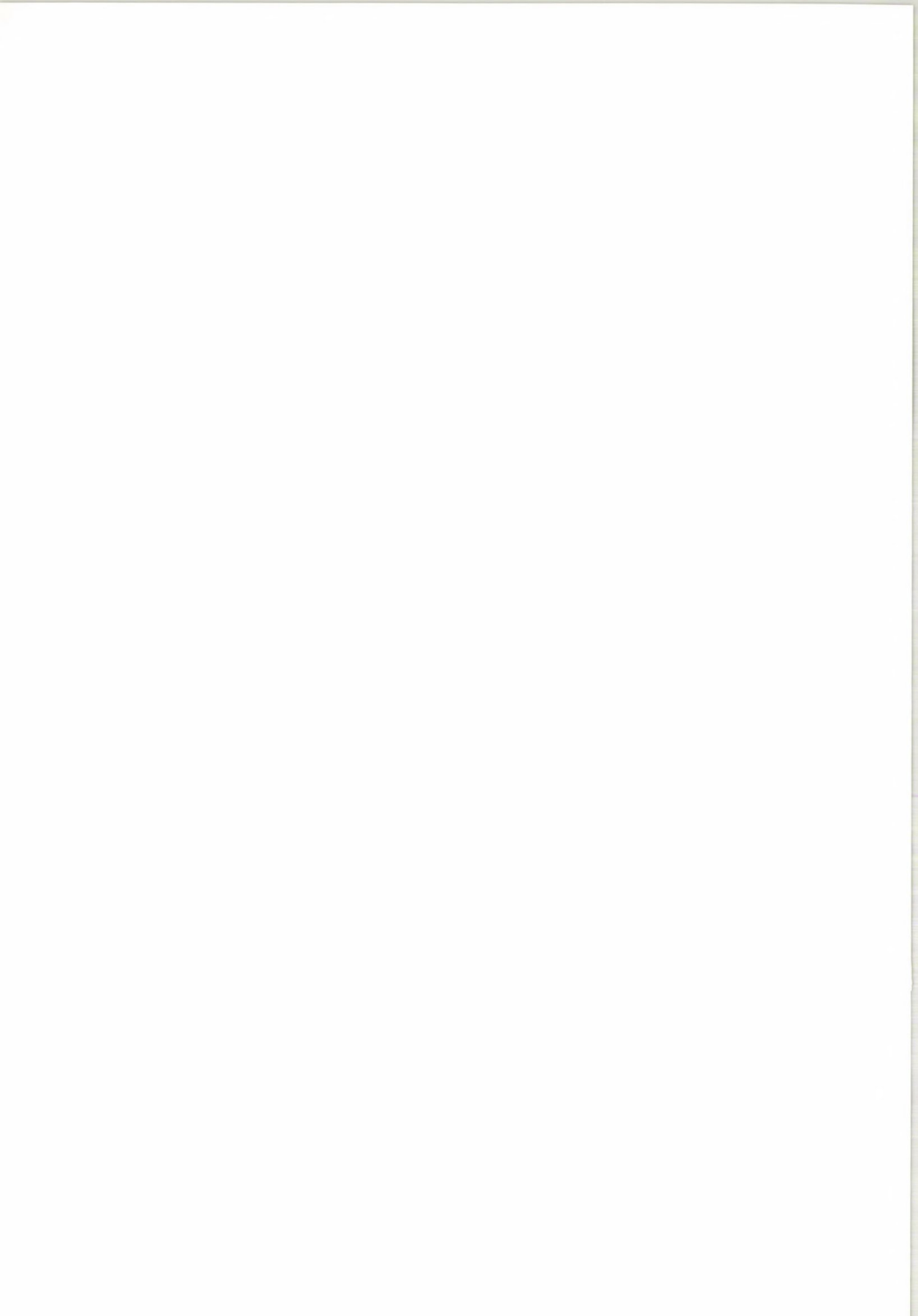
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ADJUVANT EFFECT OF STREPTOMYCIN ON ORAL IMMUNIZATION WITH SHIGELLA ANTIGENS IN THE MOUSE

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(Received August 1, 1966)

Summary. The immunogenic effect of living or formalinized bacteria and Boivin extract has been found to increase by 2 to 3 exponents if the mice had been treated with streptomycin prior to oral vaccination. A similar effect was observed when the antibiotic was given simultaneously with the antigen. The adjuvant effect showed a close association with the dose of streptomycin.

In agreement with other authors [2, 3] we have demonstrated [1] that the susceptibility of mice to *Salmonella enteritidis* infection increased by 4 to 5 exponents after oral administration of streptomycin. Similar results were obtained for experimental infection of mice with *Shigella*. The number of *Shigella* cells necessary for inducing a prolonged, symptomless excretion of the agent in streptomycin-treated mice was lower by 4 to 5 exponents than the dose needed to bring about the same effect in untreated animals [4].

In a series of experiments [5] we examined the immunogenicity of living vaccines prepared from avirulent *Shigella* strains. In the course of these investigations the idea arose that the adjuvant effect of streptomycin may be suitable for promoting the immunogenic effect of avirulent organisms. This has prompted us to study the effect of streptomycin on non-viable antigens.

Materials and methods

Vaccines. Living vaccine was prepared from *E. coli* × *Sh. flexneri* 2a hybrid strain M22—18 × 16 of the WALTER REED research team [6]. The strain retained its capacity to penetrate. For the present experiments we isolated a streptomycin resistant mutant from this organism. Virulent *Sh. flexneri* 2a strain 2aV and *Sh. flexneri* 3 strain RV₁ were used as corpuscular vaccines and as Boivin extracts.

Corpuscular vaccines were prepared by killing virulent bacteria with 0.3 per cent formalin and adjusting the density by use of the Human substandard of the N. I. H. 10T standard (Dr. Joó). Antigenicity of Boivin extracts was expressed in bacterial count equivalents by comparing the preparations to bacterial vaccines on the basis of haemagglutination inhibition [7].

Streptomycin treatment. Streptomycin was administered orally through a metal tube, in doses of 2 to 10 mg on each of two days preceding immunization, or in single doses simultaneously with the vaccine. Mice immunized with different doses of vaccine received always the same amount of streptomycin. The animals were not isolated during streptomycin treatment.

Immunization. Groups containing 8 to 10 mice each were immunized by graded doses of vaccine administered orally through a gastric tube. Each group received 5 equal doses at 3 day intervals. The animals were infected 5 days after the last immunizing dose.

Testing of active protection by the mouse shigellosis model. Mice kept under sterile conditions [4, 5], were given 10 mg streptomycin orally on each of two subsequent days. On the third day they were infected with a few cells of a virulent clone. In order to show shigella excretion, 3 days after infection the faeces were seeded onto eosin methylene blue agar.

Testing of active protection by the mucin technique. Immunized mice were infected intraperitoneally with virulent organisms suspended in 5 per cent mucin. The doses were titrated in preliminary virulence tests so as to correspond to 100 to 1000 LD₅₀. The number of surviving animals was recorded after 3 days observation.

Statistical analysis. LD₅₀ and ED₅₀ values were estimated as described by KÄRBER [8].

Results

The starting point of the present experiments was that if the infectiveness (penetration or invasiveness) of shigellae was influenced by streptomycin, the drug might exert a similar effect in the case of an avirulent organism which had retained its penetrating capacity, and then, as a result of the increased invasiveness, more bacteria would gain entrance to the intestinal wall and increase the immunogenic effect.

In order to confirm the hypothesis, two groups of mice were immunized with hybrid strain M22—18×16; animals in one of these groups received 10 mg streptomycin on each of two days preceding immunization. Two similar groups (streptomycin-treated and untreated mice) were immunized with formalinized cells. The results are shown in Table I.

Table I

Effect of streptomycin oral onimmunization with living and killed bacteria
(Challenging dose, *Sh. flexneri* 2aV ~ 5 cells)

Vaccine	Dose (total no. of cells)	Excreter/infected mice	ED ₅₀ (cells)
M22—18×16 living hybrid strain, untreated mice	5×10 ⁸	0/8	5×10 ⁶
	5×10 ⁷	1/8	
	5×10 ⁶	3/8	
M22—18×16 living hybrid strain, streptomycin-treated mice	5×10 ⁸	0/8	<5×10 ⁵
	5×10 ⁷	0/8	
	5×10 ⁶	0/8	
<i>Sh. flexneri</i> 2aV formalinized vaccine, untreated mice	5×10 ⁸	2/8	5×10 ⁷
	5×10 ⁷	4/8	
	5×10 ⁶	6/8	
<i>Sh. flexneri</i> 2aV formalinized vaccine, streptomycin-treated mice	5×10 ⁸	0/8	<5×10 ⁶
	5×10 ⁷	0/8	
	5×10 ⁶	2/8	
Control		6/8	

The data presented in Table I have confirmed our hypothesis. Although the results obtained in streptomycin-treated and untreated animals were not significantly different, the immunogenicity of the hybrid strain certainly increased when it had been administered with streptomycin. Unexpectedly, streptomycin exerted a definite adjuvant effect on formalinized vaccines.

In subsequent experiments we studied the anti-infective immunity of animals which had been treated with streptomycin and then immunized with a larger scale of graded doses of formalinized bacteria. Streptomycin was given in two different manners. The animals were treated partly as in previous experiments (10 mg streptomycin on each of two days prior to immunization), partly by giving 10 mg streptomycin simultaneously with the vaccine. Immunized animals not treated with antibiotic and non-immunized animals served as controls. The results are summarized in Table II.

These data also confirm the adjuvant effect of streptomycin. In mice not treated with streptomycin the ED_{50} value was represented by the usual 4×10^7 cells; in streptomycin-treated animals this value was lower by approximately 3 exponents (3×10^4 or 5×10^4 cells). The experiment indicated that

Table II

Immunogenicity of formalinized Sh.flexneri cells in mice treated with streptomycin prior to, and simultaneously with, vaccination

(Challenging dose, *Sh.flexneri* 2aV ~ 6 cells)

Mode of streptomycin treatment	Dose of vaccine (total no. of cells)	Excreter/infected mice	ED_{50} (cells)
10 mg streptomycin on each of 2 days before vaccination	10^8	0/9	5×10^4
	10^7	0/10	
	10^6	0/10	
	10^5	2/10	
	10^4	7/9	
10 mg streptomycin simultaneously with vaccination	10^8	0/9	3×10^4
	10^7	0/8	
	10^6	0/10	
	10^5	3/9	
	10^4	5/10	
Untreated animals	10^9	0/9	4×10^7
	10^8	2/9	
	10^7	6/9	
	Control	6/9	

Table III

Relationship between dosage of streptomycin and its adjuvant effect on vaccination

(Vaccine, *Sh. flexneri* 2a formalized cells;
challenging dose, *Sh. flexneri* 2aV ~ 4 cells)

Dose of streptomycin*	Dose of vaccine (total no. of cells)	Excreter/infected mice	ED ₅₀ (cells)
10 mg	10 ⁷	0/10	
	10 ⁶	1/10	$\sim 10^5$
	10 ⁵	4/10	
2 mg	10 ⁷	1/10	
	10 ⁶	2/10	4×10^5
	10 ⁵	6/10	
0.4 mg	10 ⁷	3/10	
	10 ⁶	5/10	10^6
	10 ⁵	7/10	
Untreated animals	10 ⁹	0/10	
	10 ⁸	2/10	$\sim 2 \times 10^7$
	10 ⁷	4/10	
	Control	7/10	

* Amount of streptomycin given simultaneously with each vaccination.

pre-treatment with the antibiotic is unnecessary; the same effect is obtained when streptomycin is given simultaneously with the vaccine.

Next, the relation between the concentration of streptomycin and the adjuvant effect was studied.

On the basis of preliminary experiments, streptomycin was given in doses of 10, 2 and 0.4 mg. Results are shown in Table III; they revealed a close connexion between the dose and the adjuvant effect of streptomycin. A decrease of the dose resulted in a proportional, although not linear, decrease in the drug's adjuvant effect.

In view of the well-demonstrable adjuvant effect of streptomycin on the immunogenicity of formalized vaccine, it seemed desirable to examine the effect of the antibiotic on vaccination with Boivin extract. Owing to the better absorption of Boivin extract, oral immunization with it is more effective than with bacterial vaccines [5]. Thus differences in the molecular dimensions of the vaccine, besides other, unknown factors, may influence the adjuvant effect of streptomycin.

Streptomycin-treated and untreated control mice were immunized with graded amounts of Boivin extract then infected orally. Results are presented in Table IV.

Table IV

Adjuvant effect of 2 mg streptomycin on immunogenicity of Boivin extract
(Challenging dose, *Sh.flexneri* 2aV ~ 7 cells)

Antigen	Dose of vaccine*	Excreter/infected mice	ED ₅₀ (cells)
Boivin extract, untreated mice	5×10^9	0/10	$\sim 5 \times 10^6$
	5×10^8	0/10	
	5×10^7	1/10	
	5×10^6	4/9	
Boivin extract, mice treated with 2 mg streptomycin simultaneously with vaccination	5×10^7	0/10	$\sim 5 \times 10^4$
	5×10^6	0/9	
	5×10^5	2/10	
	5×10^4	3/10	
	Control	6/8	

* Total dose given in 5 parts; dose of Boivin antigen is expressed in cell count equivalents.

Table IV demonstrates that streptomycin improves the immunogenicity of colloidal antigens. The degree of adjuvant effect was similar to that shown for formalinized vaccines in that ED₅₀ values were lower by about 2 exponents for mice given 2 mg doses of streptomycin.

The above results were obtained by use of the mouse shigellosis model, which, as indicated by our observations, demonstrates massive immunity. It was therefore necessary to examine the phenomenon with a different technique on a different model. Streptomycin-treated and untreated mice were immunized orally with graded equivalent doses of formalinized bacteria and Boivin extract. The animals were challenged by the mucin technique. Results are shown in Table V.

Table V

Examination of the adjuvant effect of streptomycin by the mucin technique
(Challenging dose, *Sh.flexneri* 3—100 LD₅₀)

Antigen	Untreated mice		Mice treated with 2 mg streptomycin	
	ED ₅₀ (cells)	RE	ED ₅₀ (cells)	RE
<i>Sh.flexneri</i> 3 formalinized vaccine	5×10^7	1	4×10^6	11
<i>Sh.flexneri</i> 3 Boivin extract	$2 \times 10^{7**}$	3	$2 \times 10^{6**}$	30

* LD₅₀ = 2×10^3 cells.

** Dose of Boivin extract is expressed in cell count equivalents.

It is evident from Table V that the adjuvant effect of streptomycin on immunogenicity could be demonstrated also by the mucin technique for both corpuscular and colloidal vaccines. As with the mouse shigellosis model, the order of magnitude of effective doses determined in these experiments, was only relative. The data indicate that streptomycin increased about 10-fold the immunogenicity of both kinds of antigen.

Discussion

It has been shown that streptomycin increases not only the infectivity and consequently the immunogenicity of living microorganisms capable of actively penetrating the intestinal wall, but also the immunogenicity of killed bacteria and colloidal extracts.

The mechanism of the adjuvant effect of the antibiotic is unknown. As regards salmonella infections, it is generally believed that an impairment of the intestinal flora by the antibiotic is responsible for the phenomenon [9–12]. Our experiments and the studies of BOHNHOFF *et al.* [10] indicate that the course of parenteral infection with salmonellae is not influenced by streptomycin. BOHNHOFF *et al.* [10] have shown that streptomycin leaves the intensity of passage unaffected and causes no demonstrable clinical or morphological changes. On the other hand, they also found that the adjuvant effect of streptomycin shows a linear relationship with the increase of pH and decrease of fatty acid content in the colon [13]. It has been revealed [1, 14] that the original resistance can be restored by feeding faeces but not by giving *E. coli* alone or in combination with other constituents of the faecal flora. Thus we are compelled to conclude that there is no sufficient explanation for the adjuvant effect of streptomycin in infections caused by *Salmonella*.

The same holds for experimental infection of animals with *Shigella*. Our [5] and other authors' [15–17] data indicate that streptomycin and perhaps other antibiotics (erythromycin, chloramphenicol) considerably increase the chance of, or are essential for, a successful shigella infection of mice and guinea pigs. Feeding of *E. coli* seems, however, to eliminate this increased susceptibility.

The present findings have thrown new light on the mode of action of streptomycin on antigens absorbed in the intestinal tract. It seems obvious to assume a relationship between the enhancement of oral salmonella and shigella infections by streptomycin and the adjuvant effect of the antibiotic on immunogenicity. The possibility of some different mechanism, however, cannot be excluded. The first question arising from this consideration is, whether or not corpuscles of the size of bacterial cells are absorbed from the intestinal tract. The answer is in the positive, since LODENKÄMPER [18],

VOLKHEIMER [19] and RAETTIG [20] uniformly showed that corpuscles even larger than bacteria can pass through the intestinal wall by "paracellular immigration". It can, however, hardly be supposed that this passive penetration should be promoted by an impairment of all or some members of the intestinal flora. The finding that streptomycin given simultaneously with the antigen is as effective as on previous administration does not support this explanation. Results as to the close relationship between streptomycin concentration and adjuvant effect, as well as the fact that when mice are not kept under sterile conditions even repeated doses of streptomycin cause no reduction in the intestinal flora, are also inconsistent with the above explanation.

Whether streptomycin or other substances may or may not enhance the penetration of macromolecules and corpuscles into the intestinal wall, should be the subject of further experiments.

Another experimental task is to elucidate how the adjuvant effect of streptomycin could be used for increasing the immunogenic effect of oral vaccines.

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THE FERTILITY OF *ESCHERICHIA COLI* O101 : K(A)30 : — STRAIN 18A10 IN MATING WITH *ESCHERICHIA COLI* K12

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(Received August 18, 1966)

Summary. *E. coli* strain 18A10 characterized by antigenic structure O101 : K(A)30 : — and pathogenicity to suckling mice, supplied mutants with suitable markers. Conjugation analysis with *E. coli* K12 showed that strain 18A10 corresponded to mating type F⁻. Of the 6 markers examined (proline, threonine, lactose, methionine, histidine and streptomycin) 2 were transferred by conjugation (proline and threonine). Methionine, histidine and streptomycin markers were not transferable. The lactose region was transferred with low frequency and the recombinants were partly unstable. It has therefore been concluded that, although *E. coli* 18A10 is fertile with K12, the two strains are genetically highly inhomologous.

According to ØRSKOV and ØRSKOV [1] *E. coli* strains K12, B and C, which have extensively been used in studies on *E. coli* genome, are R-mutants and consequently inadequate for immunogenetic investigations. To this consideration it may be added that these cultures are even less suitable for studies on pathogenicity or virulence.

Experiments with *E. coli* 18A10 have been described previously [2—4]. MOLL and INGALSBE, who had isolated this strain, demonstrated its pathogenicity for suckling mice [5]. FRIEDMAN and HALBERT have confirmed this finding [6]. Clinical and pathomorphological observations and the fact that pathogenicity was restricted to a certain age-group, pointed to an analogy between this strain and the *E. coli* strains associated with infantile diarrhoea [2]. Further studies showed that the type culture of antigenic group 09 : K(A)30 (strain E69), which contained a K antigen identical with that of strain 18A10, was also pathogenic for suckling mice [3]. From these observations it has been concluded that the virulence of, or some virulence factors in, strain 18A10 may be associated with the synthesis of antigen K(A)30.

In the first step of genetic analysis the fertility of this strain in mating with *E. coli* K12 has been examined.

Materials and methods

Strains. In addition to *E. coli* parent strain 18A10 (antigenic structure: O101 : K(A)30 : —) and its mutants (Table II), the following *E. coli* clones were employed: W 677 and W 1607 F⁻, 58—161 F⁺—, Hfr H, Hfr C, AB311 and AB312 Hfr.

E. coli K12 strains were kindly supplied by Dr. L. ALFÖLDI (Szeged, Hungary), Drs. F. and I. ØRSKOV (Copenhagen, Denmark), and Dr. T. M. LACHOWITZ (Wrocław, Poland).

Culture media. DAVIS' minimal medium [7] solidified with 2 per cent washed agar was used. Supplementations with amino acids, bases and vitamins at concentrations of 20 $\mu\text{g}/\text{ml}$ were performed. Sometimes streptomycin was added at suitable concentrations. In order to show the fermentation of lactose, this sugar was substituted for glucose in the medium and 16 ml 1 : 500 bromothymol blue solution was added. Lactose fermentation was examined on Endo medium.

Mutagenic agents and isolation of mutants. Mutation and reversion of K antigen were examined on meat infusion agar without the use of mutagenic agents and selective methods.

In order to facilitate the selection of other mutants the culture was irradiated with ultraviolet light (Tungsram U.V. lamp, 20 W, 50 cm) or treated with HNO_2 and NH_2OH . Some mutants were isolated by use of the membrane filter technique described by MATNEY and GOLDSCHMIDT [8] and selection with penicillin (200 I.U./ml). Most mutants were selected without penicillin by the replica plating technique of LEDERBERG [9].

Streptomycin resistant mutants were isolated on gradient plates as described by SZYBALSKI and BRYSON [10].

ONPG test. Beta-galactosidase activity in lactose negative mutants was examined as described by LE MINOR [11].

Serological checking of strain 18A10 and its mutants was performed by slide agglutination in O and OK sera.

Conjugation. Eighteen hour broth cultures of the donor and recipient strains were diluted 1 : 20 with pre-heated sterile broth and incubated further for 2 hours. Mating was performed by mixing 1 part of the donor culture with 10 parts of the recipient culture and incubating the mixture for 2 hours at 37° C. After washing twice, the suspension was plated on minimal and partially supplemented minimal agar. Results were read after 48 hours incubation.

Statistical methods. Mutation rate (per cell per division cycle) was calculated for K- mutants and revertants. The number of recombinants was expressed as the ratio of recombinant to donor cells.

Results

1. K^- mutation and reversion in strain 18A10. As K antigen represented an easily distinguishable marker, it was desirable to examine its spontaneous mutation. The results are shown in Table I.

Table I

K^- mutation and reversion in *E. coli* O101 : K(A)30 : — strain 18A10

K^- mutation

Experiment	No. of colonies examined	No. of K^- mutants	Mutation rate
1	4437	93	4.7×10^{-6}
2	3681	186	1.3×10^{-5}

K^+ reversion

No. of colonies examined	No. of K^+ revertants	Reversion rate
33 060	1	$\sim 1.1 \times 10^{-9}$

It is seen that the rate for K⁻ mutation was high (1.3×10^{-5} to 4.7×10^{-6}). Reversion was infrequent ($\sim 1.1 \times 10^{-9}$).

Isolation of mutants for genetic analysis. U.V. irradiation and the membrane filter technique yielded some mutants. Owing to the bactericidal effect of irradiation, the mutation rate was very low. This technique was also unfavourable for the isolation of polyauxotrophic mutants. The results were equally unsatisfactory with MATNEY's technique based on partial completion of the medium and thus on differences in the size of colonies.

The usefulness as a bacterial mutagenic agent of HNO₂, although it is generally recommended for the treatment of released bacteriophages [12], purified DNA [13] and RNA [14], was also examined. Some mutants were isolated, but similarly to U.V. irradiation, the substance was bactericidal even when the concentration had been decreased to 0.4 M and the time of exposure to 5 minutes.

Most of our mutants were obtained with hydroxylamine treatment. The conditions were optimal in a 4 M NH₂OH · HCl solution to which an equal part of broth culture had been added. Exposure for 1 hour was sufficient. The mutants are shown in Table II.

Mutants 3 (lac⁻), 4, 5 (met⁻), 13 (pro⁻) and 21 (his⁻) were obtained with U.V. irradiation. HNO₂ treatment yielded mutants 12 (lac⁻), 22, 23 (his⁻). The remaining mutants were isolated after exposure to hydroxylamine. Streptomycin resistant and dependent mutants were selected on gradient plates.

3. *Mating type of E. coli 18A10.* Fertility of *E. coli* 18A10 with *E. coli* K12 was examined by conjugating our proline-requiring mutant 13 with K12 F⁻ (W677), F⁺ (58—161) and Hfr H. Repeated examinations, in which

Table II

Mutant clones selected from E. coli 0101 : —K(A)30 : strain 18A10

1. K ⁺ str ^r	13. pro ⁻	21, 22, 23. his ⁻
2. K ⁻ str ^r	14. pro ⁻ lac ⁻	24. his ⁻ lac ⁻
3. lac ⁻	15. pro ⁻ cys ⁻ lac ⁻	25. his ⁻ met ⁻ lac ⁻
4, 5. met ⁻	16. pro ⁻ B12 ⁻ lac ⁻	26. his ⁻ lac ⁻ str ^d
6, 7. met ⁻ lac ⁻	17. pro ⁻ met ⁻ lac ⁻	27. his ⁻ met ⁻ lac ⁻ str ^r
8, 9. met ⁻ thr ⁻	18. pro ⁻ met ⁻ lac ⁻ str ^r	
10, 11. met ⁻ thr ⁻ lac ⁻	19. pro ⁻ met ⁻ thr ⁻ lac ⁻	
12. met ⁻ thr ⁻ lac ⁻ str ^r	20. pro ⁻ met ⁻ thr ⁻ lac ⁻ str ^r	

Abbreviations: K⁺ and K⁻ = phenotypes with and without K antigen; str^r and str^d = streptomycin resistant and dependent mutants; lac⁻ = lactose-non-fermenting mutants; met⁻, thr⁻, pro⁻, cys⁻, B12⁻ and his⁻ = methionine, threonine, proline, cysteine, vitamin B12 and histidine dependent mutants.

Table III

Mating of mutant 20 (*pro*⁻*met*⁻*thr*⁻*lac*⁻*str*^r) of *E. coli* 18A10 with *E. coli* K12 Hfr C (*met*⁻)

Strains	Revertant/ml		Recombinant/donor cell		
	<i>pro</i> ⁺	<i>thr</i> ⁺	<i>pro</i> ⁺ <i>thr</i> ⁺ <i>lac</i> ⁺	<i>pro</i> ⁺ <i>thr</i> ⁺	<i>pro</i> ⁺
Hfr C × "20"	.	.	5.6×10^{-7} *	1.5×10^{-6}	5.3×10^{-6}
"20"	<1	<1	—	—	—

* Segregation of *lac*⁻ colonies from *lac*⁺ colonies (1.2×10^{-3}).

the *pro*⁺ reversion of mutant 13 was taken into consideration, indicated that only the mating of mutant 13 with strain Hfr H yielded recombinants.

4. *Mating of E. coli* 18A10 with strains K12 Hfr. As the number of colonies obtained in the mating of mutant 13 with strain Hfr H was considerably higher than the number of revertant colonies, the frequency of proline information transfer was estimated to $1.2-6.2 \times 10^{-3}$ per donor cell.

This finding has been confirmed by experiments with a polyauxotrophic mutant 20 (*pro*⁻*met*⁻*thr*⁻*lac*⁻*str*^r). Data for the mating of this mutant with strain Hfr C are presented in Table III.

In addition to control examinations for reversion, the mixture of strain Hfr and mutant 20 was streaked on minimal medium supplemented with methionine and methionine + threonine. Both media contained 400 µg/ml streptomycin per ml. Colonies from these media were replica plated on a minimal medium containing methionine, threonine, lactose and brilliant green. In this manner *pro*⁺, *pro*⁺ + *thr*⁺ and *pro*⁺ + *thr*⁺ + *lac*⁺ recombinant classes could be observed. The recombinants acquired more often the proline than the threonine region. As in strain Hfr C lactose is the lead marker, the low frequency of the *pro* + *thr* + *lac* recombinant group was striking. This finding may be explained by the fact that *lac*⁻ segregants occurred among *lac*⁺ recombinant colonies (1.2×10^{-3}).

In further experiments the transfer by conjugation of the methionine marker was examined. Strain AB 312 Hfr served as a donor, mutants 17 (*pro*⁻*met*⁻*lac*⁻) and 5 (*met*⁻) of strain 18A10 and strain W1607 (*F*⁻*met*⁻*lac*⁻*str*^r) as recipients. Some of the results are shown in Table IV.

Table IV shows that the methionine marker was successfully transferred to K12 *F*⁻ but not to mutants of strain 18A10 ($<1.3 \times 10^{-8}$).

Transfer of the *lac* region was examined with strain Hfr C and mutant 18 (*pro*⁻*met*⁻*lac*⁻*str*^r); strain W1607 (*F*⁻*met*⁻*lac*⁻*str*^r) was used as a control. Our mutant, on the basis of ONPG positivity, was regarded as a *Y*⁻ mutant; it yielded no *lac*⁺ revertants ($<6.5 \times 10^{-5}$). Counterselection of the donor was performed by streaking after conjugation a 10^{-5} dilution of the washed suspension on Endo agar containing 400 µg streptomycin per ml. Results are shown in Table V.

Table IV

Transfer of the methionine marker from E. coli K12 Hfr to E. coli 0101 : K(A)30 : — strain 18A10

Experiment	Donor strain (markers)	Recipient strain (markers)	Recombinant/donor cell
1	AB 312 (thr ⁻ leu ⁻ str ^r)	“17” pro ⁻ met ⁻ lac ⁻ W1607 (met ⁻ lac ⁻ str ^r)	$<4 \times 10^{-7}$ 1.3×10^{-5}
2	AB 312 (thr ⁻ leu ⁻ str ^r)	“5” (met ⁻) W1607 (met ⁻ lac ⁻ str ^r)	$<1.3 \times 10^{-8}$ 1.9×10^{-5}
3	AB 312 (thr ⁻ leu ⁻ str ^r)	“5” (met ⁻) W1607 (met ⁻ lac ⁻ str ^r)	$<1.5 \times 10^{-8}$ 1.7×10^{-5}

While lactose fermentation was often transferred to *E. coli* K12 F⁻ (4.5×10^{-3} — 8.6×10^{-2} per donor cell), transfer was unsuccessful in the case of mutant 18 (lac⁻) ($<3.8 \times 10^{-4}$). This finding agrees with data obtained for the proline and threonine regions, which were transferred at low frequency and even exhibited segregation as to the lactose marker.

Similar results were obtained for the transfer of histidine and streptomycin markers (<2.3 — $<5 \times 10^9$).

Table V

Transfer of lactose marker from E. coli K12 Hfr to E. coli 0101 : K(A)30 : — strain 18A10

Experiment	Donor strain (markers)	Recipient strain (markers)	Recombinant/donor cell
1	Hfr C (met ⁻)	“18” (pro ⁻ met ⁻ lac ⁻ str ^r) W1607 (met ⁻ lac ⁻ str ^r)	$<9 \times 10^{-4}$ 4.5×10^{-3}
2	Hfr C (met ⁻)	“18” (pro ⁻ met ⁻ lac ⁻ str ^r) W1607 (met ⁻ lac ⁻ str ^r)	$<3.8 \times 10^{-4}$ 8.6×10^{-2}

Discussion

The present experiments were undertaken because of the possible usefulness in genetic analysis of an organism belonging to the same genus as *E. coli* K12. It was expected that difficulties encountered in the genetic study on the virulence of *Salmonella* [15] and *Shigella* [16, 17] could be overcome by using an *E. coli* strain. The examinations had two basic requirements, namely isolation of mutants carrying suitable markers and the fertility of these mutants in crosses with *E. coli* K12.

First of all the spontaneous loss and reversion of K antigen production had to be considered. In this respect our findings corresponded in order of magnitude to the data of SCHLEGEL-OPRECHT and FEY [18].

It is remarkable that HNO_2 was successfully applied for the isolation of bacterial amino acid dependent and fermentation mutants. In effectiveness this substance was inferior to other chemical mutagenic agents. The finding awaits confirmation, since the mutants obtained may have been produced spontaneously, without the influence of HNO_2 .

Examination of the fertility of the mutants with *E. coli* K12 gave the following results. In mating with *E. coli* K12 F^- , strain 18A10 did not act as a donor; with *E. coli* K12 Hfr it behaved, at least in certain regions, as a recipient. Accordingly, strain 18A10 corresponded to an F^- culture.

Among the 6 markers examined, pro and thr were transferred to our strain. Transfer of the lac⁺ marker was unstable and infrequent. Methionine and histidine synthesis and streptomycin resistance could not be transferred.

LEDERBERG *et al.* [19] studied the interfertility of *E. coli* K12 and other *E. coli* strains. They found that 1/20 of the strains were fertile. ØRSKOV and ØRSKOV [20] showed that 30 per cent of about 200 type determined strains of *E. coli* were fertile with K12 Hfr strains. In reciprocal matings LEDERBERG [21] revealed that for *E. coli* K12 F^- only 1/40 of *E. coli* strains acted as donors. According to GROSS [22] infertility may be due to various factors: (1) Inability of cellular conjugation; (2) poor homology of the genetic material; (3) other factors, e.g. colicin production.

For the partial infertility encountered in our experiments the poor homology of certain regions seems to be responsible. The homology of the proline and threonine regions in our strain and in *E. coli* K12 is probably sufficient to allow recombination. In contrast, owing to the high degree of inhomology, recombination is inhibited in the histidine, methionine and streptomycin regions. The moderate inhomology in the lactose region allows a rare recombination and/or perhaps a partial diploidization.

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SEGREGATION OF R FACTOR IN SHIGELLA AFTER ACRIDINE ORANGE TREATMENT

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(Received August 1, 1966)

Summary. Acridine orange treatment caused segregation in a sulphonamide-streptomycin-chloramphenicol-tetracycline resistant *Sh. sonnei* strain. The segregant clones were sensitive to streptomycin-chloramphenicol-tetracycline or to tetracycline. Sulphonamide resistance, which could not be deleted, has been attributed to chromosomal origin or to recombination between the sulphonamide determinant and the homologous chromosome. The results pointed to the presence of two linkage groups in the R factor. Serial passage in acridine orange and ultraviolet irradiation yielded further data for the integration of the R factor.

In a previous study [1] it has been shown that acridine treatment results in the segregation of the R factor in *Shigella* strains. As this observation was incompatible with other data [2, 3], it seemed desirable to perform a more detailed analysis.

Materials and methods

Strains. Acridine-induced segregation or deletion was observed in *Sh. sonnei* R (phase 2) strain 549, which had been isolated from routine material. The strain was resistant to sulphonamide (Sa), streptomycin (Sm), chloramphenicol (Cm) and tetracyclines (Tc). Its R factor (R₅₄₉) was designated as *Sa.Sm.Cm.Tc*.

Two of the clones isolated after acridine treatment were analysed in detail. One of them (549/319) had lost its Tc resistance and was therefore designated as *Sa.Sm.Cm(Tc⁻)*. The other segregant became sensitive to Sm, Cm and Tc but retained its Sa resistance: *Sa(Sm⁻.Cm⁻.Tc⁻)*.

In transfer experiments the achromogenic *Serratia marcescens* strain 229, which had been used previously [1] and mutants W 1607, F⁻; W 1895, Hfr₁ of strain *E. coli* K12 were used. *E. coli* K12 strains were kindly supplied by Drs. I and F. ØRSKOV, International Escherichia Centre, State Serum Institute, Copenhagen.

Acridine treatment and isolation of segregants. *Sh. sonnei* 549 and its above-mentioned representative clones were cultured for 18 to 20 hours in broth containing 50 µg per ml of acridine orange. Suitable dilutions (10⁻⁴—10⁻⁵) of the cultures were streaked on agar plates. The young agar culture, if it contained a sufficient number of colonies, was replica plated by LEDERBERG's method [4] onto antibiotic-containing (10 to 50 µg/ml) and antibiotic-free agar plates. All clones which had failed to grow on plates with antibiotics were isolated and the loss of resistance was checked by the paper disk method.

In some experiments serial passages were made in acridine orange broth.

Induction by ultraviolet irradiation. Saline suspensions containing 10⁹ cells per ml were irradiated (Tungsram U.V. lamp, 20 W, 50 cm). The reduction in viable counts amounted to about 3 exponents. After irradiation the suspension was inoculated into acridine orange broth.

Transfer experiments. Transfer of the R factor was attempted as follows. The donor and recipient cultures were diluted 1 : 20, incubated for 2 hours, mixed at volumes 1 : 10 and reincubated for 2 hours at 37° C. Counterselection of the donor was performed with virulent phage F2, isolated and kindly supplied by Professor S. SLOPEK, Hirsfeld Institute, Wrocław. Recipient strains were counterselected on the basis of antibiotic resistance.

Determination of drug resistance. Clones not growing on plates containing antibiotics were checked for sensitivity by the paper disk method. Sulphonamide disks contained 400 µg; streptomycin, chloramphenicol, and tetracycline disks, 30 µg substance.

Some representative clones giving different zones of inhibition in the disk test were examined by the tube dilution technique using antibiotic concentrations of 0.47–500 µg/ml in 2 ml volume. The quantitative tests were performed simultaneously for all examined clones. The antibiotic dilutions were seeded so as to contain 5×10^5 cells per ml. Results were read after incubation for 48 hours.

Results

(1) *Acridine treatment of Sh. sonnei strain 549.* The result obtained after acridine treatment of the original strain was checked in repeated experiments (Table I).

From Table I it is evident that examination of more than 7000 colonies failed to show a complete deletion of the R factor. Partial deletion or segrega-

Table I
Segregation of R factor in *Sa.Sm.Cm.Tc* resistant
Sh. sonnei strain 549 after exposure to acridine orange

Experiment	No. of colonies examined	No. of R ⁻ colonies	No. of segregants	
			<i>Sa</i> (<i>Sm</i> ⁻ , <i>Cm</i> ⁻ , <i>Tc</i> ⁻)	<i>Sa. Sm. Cm</i> (<i>Tc</i> ⁻)
1	663	0	45	8
2	109	0	8	0
3	409	0	2	10
4	91	0	2	4
5	689	0	19	2
6	293	0	5	1
7	430	0	38	0
8	92	0	11	1
9	349	0	16	0
10	129	0	12	0
11	416	0	23	3
12	140	0	26	1
13	125	0	21	0
14	131	0	7	0
15	806	0	13	2
16	1029	0	14	1
17	273	0	25	7
18	289	0	76	0
19	233	0	50	1
20	391	0	69	3
Total	7087	0 (<0.01%)	482 (6.8%)	44 (0.62%)

tion of the merogenote was, however, common. Segregants having lost *Tc* resistance [*Sa.Sm.Cm(Tc⁻)*] occurred in 0.62, those having lost *Sm*, *Cm* and *Tc* resistance [*Sa(Sm⁻.Cm⁻.Tc⁻)*] in 6.8 per cent. Although the incidence of segregants varied in the different experiments, it showed a normal and not a clonal type distribution. The experiments revealed accordingly that *Sa(Sm⁻.Cm⁻.Tc⁻)* segregation was about 10 times as frequent as *Sa.Sm.Cm(Tc⁻)* segregation.

Since no *R⁻* clones had been detected, prolonged exposure by passaging in acridine broth was attempted. Culturing was performed from each passage by the replica plating technique. Results are shown in Table II.

Table II shows the effect on *R₅₄₉* factor of 6 consecutive passages in acridine broth. It is seen that no *R⁻* clones were revealed among 2251 colonies. It was interesting that during serial acridine treatment the tendency for segregation decreased gradually. Segregation *Sa(Sm⁻.Cm⁻.Tc⁻)*, which was frequent after the first exposure (9.3 per cent), appeared rarely after the third passage.

(2) *Acridine treatment of segregant clones R_{549/319} and R_{549/353}*. Two representative segregants, *R_{549/319} = Sa.Sm.Cm(Tc⁻)* and *R_{549/353} = Sa(Sm⁻.Cm⁻.Tc⁻)* were treated with acridine on repeated occasions. The experiments were performed partly to obtain *R⁻* clones from segregant *Sa(Sm⁻.Cm⁻.Tc⁻)* which retained only the sulphonamide resistance. The other purpose was to reveal further segregations in clone *Sa.Sm.Cm(Tc⁻)*. The clones were subjected to acridine treatment and U.V. irradiation combined with subsequent acridine treatment.

Repeated treatment of clone *R_{549/319}* with acridine is presented in Table III. Neither *R⁻* colonies nor further segregants were detected. The probability of the occurrence of such segregants, as follows from the number of colonies examined (3606), is less than 0.02 per cent.

Table II

Deletion and segregation of R₅₄₉ factor on serial passage in acridine broth

Passage	No. of colonies examined	R ⁻ colonies		Segregants			
		No.	%	<i>Sa(Sm⁻.Cm⁻.Tc⁻)</i>		<i>Sa.Sm.Cm(Tc⁻)</i>	
				No.	%	No.	%
1	522	0	<0.1	49	9.3	2	0.3
2	478	0	<0.2	26	5.4	0	<0.2
3	189	0	<0.5	1	0.5	0	<0.5
4	241	0	<0.4	0	<0.4	0	<0.4
5	376	0	<0.2	0	<0.2	0	<0.2
6	445	0	<0.2	1	0.2	0	<0.2

Table III

Deletion of segregant R_{549/319} Sa.Sm.Cm(Tc⁻) on acridine treatment

Experiment	No. of colonies examined	R ⁻ or segregant colonies	
		No.	%
1	502	0	<0.2
2	634	0	<0.1
3	1198	0	<0.08
4	547	0	<0.1
5	725	0	<0.1
Total	3606	0	<0.027

The result of acridine treatment after U.V. irradiation is shown in Table IV. Examination of 478 colonies indicated that attempts at deleting the R factor were unsuccessful. Some *Sa(Sm⁻.Cm⁻.Tc⁻)* segregants were isolated.

Acridine treatment of clone R_{549/353} *Sa(Sm⁻.Cm⁻.Tc⁻)* yielded the results presented in Table V. The data indicate that not R⁻ (sulphonamide sensitive) clones were isolated (<0.029 per cent).

U.V. irradiation and subsequent acridine treatment of this clone, similarly to the corresponding experiments with clone R_{549/319}, yielded no R⁻ colonies (Table VI).

(3) *Conjugation experiments.* In order to show whether *Sa* resistance, which could not be deleted by acridine treatment, is or is not an episome-carried factor, transfer of this character was attempted by conjugation. These experiments failed to give positive results with any of the recipients. Transfer of other resistance determinants was likewise unsuccessful with either the original strain or its segregants acting as donors.

Corresponding experiments with the same strain in the previous study [1] yielded an equally negative result.

Antibiotic resistance. As mentioned above, all clones indicated by replica plating to have been depleted of their resistance factor, were checked by the paper disk method. As this kind of testing is inadequate for the quantitative

Table IV

Deletion of segregant R_{549/319} Sa.Sm.Cm(Tc⁻) on combined U.V. and acridine treatment

No. of colonies examined	R ⁻ colonies		Segregants <i>Sa(Sm⁻.Cm⁻)</i>	
	No.	%	No.	%
478	0	<0.2	3	0.6

Table V*Deletion of segregant R_{549/353} Sa(Sm⁻.Cm⁻.Tc⁻) on acridine treatment*

Experiment	No. of colonies examined	R ⁻ colonies	
		No.	%
1	550	0	<0.1
2	492	0	<0.2
3	665	0	<0.1
4	1319	0	<0.07
5	421	0	<0.2
Total	3447	0	<0.02

Table VI*Deletion of segregant R_{549/353} Sa(Sm⁻.Cm⁻.Tc⁻) on combined U. V. and acridine treatment*

Experiment	No. of colonies examined	R ⁻ colonies	
		No.	%
1	127	0	<0.7
2	933	0	<0.1
3	611	0	<0.1
Total	1671	0	<0.06

Table VII*Quantitative antibiotic resistance of clones having lost resistance determinants*

"Discrepant"* Sm sensitive clones	Sm resistance μg/ml	"Discrepant" Cm sensitive clones	Cm resistance μg/ml	"Discrepant" Tc sensitive clones	Tc resistance μg/ml
3	1.9	179	0.95	58	15.6
16	0.95	191	1.9	66	15.6
27	1.9	243	1.9	161***	15.6
151	1.9	308	0.95	331	15.6
161	1.9	320	1.9	353	15.6
179	1.9	366	1.9	466	15.6
				475***	15.6
Original** R ₅₄₉	250.0		250.0		125.0

* Representative clones were selected by the paper disk sensitivity test on the basis of the diameter of inhibition zones.

** Sa resistance, > 1000 μg/ml.

*** Sa.Sm. Cm(Tc⁻) segregants; the other clones are Sa(Sm⁻.Cm⁻.Tc⁻) segregants.

determination of sensitivity, some clones displaying different zones of inhibition were examined by the tube dilution technique.

The parent strain was resistant to $> 1000 \mu\text{g/ml}$ of sulphonamide 250 $\mu\text{g/ml}$ of streptomycin and chloramphenicol, and 125 $\mu\text{g/ml}$ of tetracycline.

Table VII shows the degree of resistance obtained for various clones. It is evident that the segregants were susceptible to approximately uniform levels of the antibiotics and, as far as it could be concluded from the small number of examinations, no segregation occurred in the different resistance determinants.

Discussion

WATANABE and FUKASAWA have shown [5] that the R factor, similarly to other episome-carried elements, can be eliminated in *Shigella* and *Escherichia*. The R factor tends to segregate spontaneously [5]; acridine treatment causes no segregation [2, 3].

In the present experiments, acridine treatment yielded no R^- (sulphonamide sensitive) clones and U.V. irradiation prior to acridine treatment gave similarly negative results. This finding may be explained by the chromosomal origin of the Sa^r determinant on the one hand, and by a recombination between the Sa determinant of $Sa.Sm.Cm.Tc$ R factor and the homologous chromosomal region on the other. The latter seems more probable, as the $Sa.Sm.Cm.Tc$ merogenote represents the most frequent and "classical" type of R factor. The finding that the parent strain and its segregant were highly resistant to sulphonamide ($> 1000 \mu\text{g/ml}$), even when tested on synthetic medium, also supports the latter hypothesis.

In the present experiments segregation of $Sm.Cm.Tc$ determinants occurred on acridine treatment. The segregants fell into two groups, viz. $(Sa)Sm \cdot Cm$, and Tc . This was compatible with the linear [6] and circular [7] map of the R-factor designed by WATANABE and FUKASAWA, and also agreed with the results of HASHIMOTO and HIROTA [8] who showed two linkage groups in the R factor, $Sa-Sm-Cm$, and $Tc-m$ (m = conjugation fertility).

It is of course questionable whether segregation of the R factor occurred as a result of acridine treatment or spontaneously. As serial passage of the strain in broth failed to produce segregation it may be concluded that segregation was directly or indirectly due to acridine treatment. A final conclusion, however, will only be possible when the mechanism of spontaneous segregation and integration has been elucidated.

Our data have thrown some light on the integration of the R factor. The finding that the frequency of deletion is gradually decreased on passage in acridine broth indicates that finally the population contains the integrated R factor only. The frequency of change from integrated into autonomous

state is so low that with our non-selective methods it is detected but exceptionally. As shown in the present experiments, U.V. irradiation increases the frequency of change into the autonomous state. By this technique an *Sm.Cm-Tc⁻* type segregation of the previously integrated *Sm.Cm.Tc* merogenote was demonstrated on repeated occasions.

Although few precise antibiotic sensitivity determinations were performed, preliminary selection by the paper disk technique indicated that no segregation had occurred within the various resistance determinants.

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MORGANELLA MORGANII ANTIGENICALLY RELATED TO ESCHERICHIA COLI O111 : K58(B4)

By

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(Received July 21, 1966)

Summary. (i) A close antigenic relationship has been demonstrated between *M. morganii* strain 8662/64 and *E. coli* serogroup O111.

(ii) The "b" antigen of *E. coli* O111a, b contained two partial factors ("b1" and "b2"). The O antigens of *M. morganii* strain 8662 were identical with the "a" and "b2" antigens of *E. coli* O111.

(iii) Antigen K58(B4) was also complex, as in addition to a common factor ("Ba"), O111a, b and O111a, c type strains contained less developed partial K antigens designated as "Bb" and "Bc", respectively. In addition, the presence of an agglutinogenic factor ("Bd") was demonstrated.

(iv) *M. morganii* strain 8662 contained the components "Ba" and "Bd" of the K58(B4) antigen. In thermosensitivity the K antigen of *M. morganii* showed the characters of *E. coli* B antigens. The two antigens differed functionally, as the K antigen of *M. morganii*, instead of inhibiting, increased O-agglutinability.

(v) Strain 8662 represents a new serotype which has been included in the *Morganella* antigenic schema as type strain of serogroup XXXIV.

Routine bacteriological examinations for revealing *E. coli* serotypes associated with infantile diarrhoea have been carried out in our institute since 1950. In order to detect biochemical mutants, on primary Endo agar cultures not only lactose positive, but also lactose negative colonies are routinely agglutinated. By this technique we have succeeded in isolating strain 8662/64, which agglutinated to end titre in serum O111 : K58(B4), although on polytropic medium [1] it showed the characters of *Proteus*. The present paper gives an account of the examination of this strain.

Materials and methods

Biochemical and serological examinations were carried out by the usual methods [2, 3]. *E. coli* O111a, b: K58(B4) strain Stoke W was obtained from our collection, strain *E. coli* O111a, c: K58(B4) was supplied by Dr. W. H. EWING.

Results

The biochemical reactions of strain 8662/64 were characteristic of *M. morganii* [2].

The results of serological examinations were as follows.

Table I

Cross-agglutinations between *E. coli* O111a, b : K58(B4),
E. coli O111a, c : K58(B4) and *M. morganii* 8662/64

Strains		Reciprocal agglutination titres in sera					
		O111a, b		O111a, c		8662/64	
		living	100° C	living	100° C	living	100° C
O111a, b : K58(B4)	living	320	160	640	80	320	160
	100° C	2560	10240	2560	2560	2560	2560
O111a, c : K58(B4)	living	160	80	320	160	640	160
	100° C	2560	2560	2560	5120	2560	1280
8662/64	living	2560	5120	2560	2560	2560	2560
	100° C	1280	1280	1280	1280	1280	1280

Table I demonstrates the results of cross-agglutination tests. It is seen that strain 8662/64 gave a high titre agglutination in both *E. coli* O111a, b : K58(B4) and O111a, c : K58(B4) sera. The boiled suspensions of both *E. coli* strains agglutinated to end titre in serum 8662. There was an important difference between the agglutination of living 8662 strain and living *E. coli* strains. While the living suspensions of both *E. coli* strains showed the usual low titre O agglutination, with strain 8662 this decreased agglutinability was not observed. Living cultures of 8662 agglutinated even at higher titres than the corresponding boiled bacteria. The fact that the immune serum prepared with boiled 8662 culture contained low titre antibodies against living suspensions of both *E. coli* strains indicated the presence of a thermolabile factor. This antigen, although it was seemingly closely related to antigen K58(B4), promoted the agglutination of strain 8662 instead of inhibiting it.

The O antigen analysis of our strains is presented in Table II. The intensive cross-agglutination was evidently due to antigen "111a" present in the examined three strains. Antigen "111b" was composed of two factors ("b1" and "b2"). Strain O111a, b contained both of these factors; in strain 8662 antigen "111b2" was only present. Antigen "111c" was shown only in strain O111a, c. Thus the O antigens of the *E. coli* strains can be expressed as O111a, b1, b2 and O111a, c; *M. morganii* strain 8662 is characterized by O antigens O111a, b2.

We have not determined the flagellar antigen of strain 8662. It is different from known *M. morganii* H antigens and H antigens encountered in serotypes of *E. coli* O111.

An analysis of K antigens is presented in Table III. From the results it has been concluded that according to absorption experiments 1, 2, 5 and 6, the K58(B4) antigens in strains O111a, b and O111a, c are not identical; in addition to a well-developed common antigen ("Ba") each organism possesses a specific factor ("Bb" and "Bc", respectively). Absorption experiments 3

Table II

Analysis of the somatic antigens of M. morganii strain 8662/64

Sera	Cultures used for absorption	Remaining agglutinins	Reciprocal agglutination titres with heated suspensions of		
			O111a, b	O111a, c	8662/64
O111a, b : K58(B4) 100° C	O111a, c : K58(B4) 100° C	b1, b2	320	—	80
O111a, b : K58(B4) 100° C	8662/64 100° C	b1	320	—	—
O111a, c : K58(B4) 100° C	O111a, b : K58(B4) 100° C	c	—	160	—
O111a, c : K58(B4) 100° C	8662/64 100° C	c	—	160	—
b1, b2	8662/64 100° C	b1	80	—	—
8662/64 100° C	O111a, b : K58(B4) 100° C	—	—	—	—
8662/64 100° C	O111a, c : K58(B4) 100° C	b2	160	—	160

and 4 indicated that in *M. morganii* strain 8662 a well-developed further K antigen ("Bd") is present. The "Bd" agglutinin content of sera O111a, b : K58(B4) and O111a, c : K58(B4) after cross-absorption was a paradoxical finding (experiments 5 and 6). This seemingly unilateral reaction may be explained by the fact that antigen "Bd" in *E. coli* O111 strains is functioning only as an agglutinin and thus its presence in these strains can be demon-

Table III

Analysis of the K antigen of M. morganii strain 8662/64

Experiment no.	Sera	Cultures used for absorption	Remaining agglutinins	Reciprocal agglutination titres with living suspensions of		
				O111a, b : K58(B4)	O111a, c : K58(B4)	8662/64
1	O111a, b : K58(B4) living	8662/64 living	Bb	80	—	—
2	O111a, c : K58(B4) living	8662/64 living	Bc	—	80	—
3	8662/64 living	O111a, b : K58(B4) living	Bd	—	—	640
4	8662/64 living	O111a, c : K58(B4) living	Bd	—	—	640
5	O111a, b : K58(B4) living	O111a, c : K58(B4) living	BbBd	40	—	320
6	O111a, c : K58(B4) living	O111a, b : K58(B4) living	BcBd	—	80	640
7	8662/64 living	8662/64 100° C	—	—	—	—

strated only by an agglutination of strain 8662 in the *E. coli* sera. Accordingly, the K antigens of the three strains are symbolized by the following formulae: O111a, b = Ba, Bb, (Bd); O111a, c = Ba, Bc, (Bd); 8662 = Ba, Bd, where bracketed letters stand for antigens exerting an agglutinogenic capacity only.

The last row of Table III (Experiment 7) indicates the nature of the K antigen of strain 8662. As after heating at 100° C the homologous suspension removed K agglutinins, this antigen should be classified as a B antigen, although as it has been pointed out, it differs from usual B antigens in that it fails to inhibit the O agglutination of strain 8662.

Discussion

On the basis of its characteristic biochemical properties, strain 8662/64 should be classified as a member of the genus *Morganella*. As to serological behaviour, it contains O and H antigens different from those included in the *M. morganii* antigenic schema [2]. Its antigenic analysis has been undertaken because its O and K antigens were closely related to *E. coli* antigens O111 : K58(B4).

Comparison of strain 8662 with *E. coli* type strains O111a, b and O111a, c revealed complicated relationships. In addition to antigen O111a, *M. morganii* strain 8662 contains a part of antigen "b" of *E. coli* O111a, b. Antigen "b" is composed of two partial factors: "b1" and "b2". Strain O111a, b contains both of them, while in *Morganella* 8662 factor "b2" is only present. These cultures are related to strain O111a, c by factor "a".

The relationship of K antigens is even more complicated. It has been revealed that antigen K58(B4) is also complex. Strains O111a, b and O111a, c contained specific factors "Bb" and "Bc", respectively in addition to a well developed common component "Ba." The specific K factors "Bb" and "Bc" were minor antigens of no practical importance. Strain 8662, in addition that it probably contains antigen "Ba", possesses a factor designated as "Bd". Agglutinins for the latter were shown in serum 8662 as well as in sera O111 : K58(B4). It is interesting, however, that this antigen exerts a demonstrable agglutinability and agglutinin-binding capacity only in strain 8662. In the examined *E. coli* strains it is present only as an agglutininogen, in other words, it can only be demonstrated in immune sera prepared with these strains. The K antigens of the three examined strains have been expressed with the following formulae: O111a, b = Ba, Bb, (Bd); O111a, c = Ba, Bc, (Bd); 8662 = Ba, Bd.

It was unusual that the K antigen of strain 8662 should promote instead of inhibiting O-agglutinability. This antigen therefore is functionally different from antigen K58(B4), but it resembles *E. coli* B antigens in retaining its

agglutinin-binding capacity and losing its agglutinogenicity after heating. The promoting effect of K antigen on agglutinability has already been described for the type strain of serogroup XXIX of RAUSS and VÖRÖS's *Morganella* antigenic schema [2, 4]. This strain contains antigens identical with *E. coli* antigens O112a, c : K66(B11). The K antigen of this *Morganella* strain, similarly to that of strain 8662, enhances agglutinability instead of inhibiting it [4]. Such K antigens are probably characteristic of *Morganella*.

Thus strain 8662 is the second *Morganella* serotype to contain antigens described in the genus *E. coli*. Strain 8662 corresponds to type strain of *Morganella* serogroup XXXIV [5].

Acknowledgement. The authors are indebted to Dr. W. H. EWING for supplying strain *E. coli* O111a, c.

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FIVE NEW SEROTYPES OF *MORGANELLA MORGANII*

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(Received July 21, 1966)

Summary. (i) Biochemical and serological examinations of 5 new *M. morganii* serotypes have been performed.

(ii) The inclusion in our previously elaborated antigenic schema of the 5 strains as representatives of serogroups 30—34 is proposed.

(iii) It is recommended that strains 958/O18 : H— (serogroup 18) and 979/O28 : H— (serogroup 28) should be deleted from the genus *M. morganii* and placed in the genus *R. rettgeri* without renumbering the *Morganella* antigenic schema.

In a previous paper we have given a detailed account of the biochemical and serological properties and systematic position of *M. morganii*. A diagnostic antigenic schema including 29 O groups and 3 subgroups within O group 1 has been elaborated; on the basis of 19 H antigens 57 serological types have been recognized. As in the course of these investigations we were able to identify only about 75 per cent of the collected strains as members of the above serotypes, we have called attention to the probable existence of further serotypes [1]. A subsequent investigation yielded a similar proportion of typeable strains [2].

In the present paper our previous work is supplemented with the biochemical and serological analysis of 5 new *M. morganii* serotypes and proposals are made for the modification of the diagnostic schema.

Materials and methods

Organisms. Strains C 104, D 34 and K 131 were obtained from the collection of Dr B. LÁNYI, strain 30 833/63 was received from Dr. I. D. COSTIN, and strain 8662/64 was isolated in our institute. All strains originated from faecal specimens of infants.

Biochemical reactions performed as described by KAUFFMANN are listed in Table I. Motility was examined in U tubes containing semisolid medium prepared with 0.2 per cent agar.

Serological examinations. O and H antigens were examined by our standard methods [1, 5]. The strains were tested first in polyvalent O and H sera. O and H sera were prepared as described previously [1].

Results

Biochemical reactions. The biochemical behaviour of the examined strains was characteristic of *M. morganii* [1]. They all produced indole, hydrolyzed urea, did not utilize ammonium citrate, did not produce hydrogen sulphide,

gave positive methyl red and negative Voges-Proskauer reaction. They grew in KCN-containing peptone water, produced phenylalanine deaminase and ornithine decarboxylase and did not liquefy gelatin.

Among fermentable substances only glucose, galactose, laevulose and mannose were split, with acid and gas production. On the basis of these biochemical reactions the 5 strains were classified as belonging to biotype 1 [1]. Table I presents biochemical reactions found valuable for differentiation.

Serological examinations. None of the 5 strains studied in the present work agglutinated in polyvalent O and H immune sera [1]. Therefore, O and H sera were prepared against these strains. The new sera did not agglutinate type strains of our antigenic schema. Thus it was evident that the strains contained new O and H antigens.

Cross-agglutination tests with the new sera and strains revealed no common O-antigenic components. Two strains showed a high titre H cross-

Table I

Biochemical reactions

	Designation of strain				
	C 104	D 34	K 131	30, 853/63	8662/64
Gas production	+	+	+	+	+
Lactose	—	—	—	—	—
Glucose	+	+	+	+	+
Galactose	+	+	+	+	+
Laevulose	+	+	+	+	+
Mannitol	—	—	—	—	—
Mannose	+	+	+	+	+
Adonitol	—	—	—	—	—
Inositol	—	—	—	—	—
Indole	+	+	+	+	+
Methyl red	+	+	+	+	+
Voges-Proskauer	—	—	—	—	—
Simmons' citrate	—	—	—	—	—
Urease	+	+	+	+	+
H ₂ S	—	—	—	—	—
Gelatinase	—	—	—	—	—
KCN (Braun)	+	+	+	+	+
Phenylalanine deaminase	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+
Motility	+	+	+	+	+

Table II
Relationship of H antigens of strains C 104 and D 34

Formalinized suspensions	Serum C 104 (30 : 20, 21)		Serum D 34 (31 : 21, 22)	
	Unabsorbed	Absorbed by strain D 34	Unabsorbed	Absorbed by strain C 104
C 104 (30 : 20, 21)	51,200	12,800	3,200	—
D 34 (31 : 21, 22)	3,200	—	25,600	6,400

agglutination. Cross-absorption experiments indicated an a, b—a, c relationship, that is, each of the two cultures contained a distinct type-specific factor in addition to the common H component. These experiments are summarized in Table II.

The antigenic structure of the new serotypes in conformity with our previously elaborated antigenic schema is expressed by the following formulae: C 104 = 30 : 20, 21; D 34 = 31 : 21, 22; K 131 = 32 : 23; 30 853/63 = 33 : 24 and 8662/64 = 34 : 25.

It should be noted that sera prepared with the new serotypes have already been used for typing strains with undetermined antigens. Two out of 4 such cultures of our collection belonged to serotype 30 : 20, 21; the remaining 2 strains were identified as serotypes 31 : 21, 22 and 32 : 23. Among strains collected in Japan by NAMIOKA and SAKAZAKI, 1 belonged to O group 30, 2 to O group 31 and 3 to O group 32. One strain of COSTIN belonged to serogroup O32.

Discussion

In our previous studies we have elaborated an antigenic schema for *M. morganii*, dividing 222 strains into 29 O groups, 3 subgroups and by 19 H factors into 57 serotypes. In the course of this work approximately 75 per cent of the examined *M. morganii* strains were included in these serotypes [1]. Subsequent examinations yielded similar results [2]. It was pointed out that the schema contained only the most common serotypes and a less frequent occurrence of new serotypes might be expected.

Our present studies revealed 5 new O and 6 new H antigens. No serological relationship was detected between the new O antigens; one of them, however, showed a close relationship with *E. coli* O111 : K58(B4). Analysis of this relationship has been presented in another paper [5]. Two serotypes contained a common flagellar component giving a high titre cross-agglutination between the two type strains.

Thus, as shown in Table III, our diagnostic antigenic schema has been supplemented with 5 new serotypes. It is recommended that the type strains

Table III
Supplement to the M. morganii antigenic schema

Sero-group	Type strain	Antigens		No. of strains
		O	H	
30	C 104	30	20, 21	4
31	D 34	31	21, 22	4
32	K 131	32	23	5
33	30,853/63	33	24	1
34	8,662/64	34, K2*	25	1

* Identical with *E. coli* antigens O111 B4 (K58).

should be included in the antigenic schema as representatives of serogroups 30—34. For the designation of the thermolabile antigen encountered in group 34 the symbol K2 is proposed (thermolabile antigen K1 has been recognized in O29 strains).

Finally the finding of SUASSUNA [6] should be discussed. He found that two type strains of our antigenic schema showed the characters of *R. rettgeri*, namely strains 958 (serogroup 18, O18 : H—) and strain 979 (serogroup 28, O28 : H—) fermented mannitol, salicin and inositol rapidly and adonitol slowly. These findings have been confirmed by our subsequent examinations and therefore the two strains should be deleted from the *M. morganii* schema. In order to avoid confusion, the diagnostic schema should not be renumbered. Thus in our opinion, open places remaining after the deletion of serogroups 18 and 28 should be left unfilled. This consideration is supported by the fact that several serotypes have been deleted from the *E. coli* antigenic schema and transferred to the *Citrobacter* (*E. freundii*) genus.

Acknowledgement. The authors are indebted to Dr. B. LÁNYI (National Institute of Public Health, Budapest), Dr. SH. NAMIOKA and Dr. R. SAKAZAKI (National Institute of Animal Health, Tokyo) and Dr. I. D. COSTIN (State Inspection for Hygiene, Timisoara) for supplying the cultures.

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ANTIGENIC RELATIONSHIPS BETWEEN MORGANELLA MORGANII AND DIFFERENT GENERA OF ENTEROBACTERIACEAE*

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(Received July 21, 1966)

Summary. Systematic studies have been carried out to detect O antigenic relationships between *Morganella* and other genera of the family *Enterobacteriaceae*. Several minor relationships, mostly of the a, b—a, c type, were encountered; in some strains identical O antigens were revealed. Identical O antigens were found between the species, *M. morganii* O10—*P. hauseri* O12; *M. morganii* O23—*P. hauseri* O17; *M. morganii* O8—*R. rettgeri* O10; *M. morganii* O20 : K1—*E. coli* O112a, c : K66 (B11); and *M. morganii* O34—*E. coli* O111a, b : K58(B4). A multiple relationship was demonstrated between *M. morganii* O9—*P. hauseri* O7—*Citrobacter* O14—*S. inverness*—*S. niarembé* and *S. bergen*. The importance of the relationships for practical diagnosis and antigen chemistry are discussed.

Serological relationships have been revealed between several species of the family *Enterobacteriaceae* [1, 2]. Investigations into this problem, in addition that they can be utilized in practical diagnostic work, provide a basis for the chemical analysis of antigens and are also of a theoretical importance, as they may throw light on the common origin of the family. In the literature there are numerous studies on the antigenic relationships between *Escherichia*—*Salmonella*—*Arizona*, *Salmonella*—*Citrobacter*, *Escherichia*—*Citrobacter*, *Escherichia*—*Shigella*, *Escherichia*—*Proteus*, and *Escherichia*—*P. inconstans* [1, 2, 4, 8, 10, 11, 13, 14].

Serological relationships of the genus *Morganella* have been examined less widely [2, 5, 6, 7, 9]. The present work has been designed to determine relationships of *M. morganii* O antigens of our antigenic schema [3] with O antigens of *P. hauseri* (*P. vulgaris*, *P. mirabilis*), *R. rettgeri*, *P. inconstans* (*Providencia*), *E. coli*, *Citrobacter* and *Salmonella*.

Materials and methods

Strains. Organisms used in these experiments were maintained in nutrient agar stab cultures. The strains and their sources were as follows. *M. morganii* O1—O29 (own collection); *P. hauseri* O1—O49 (Dr. B. LÁNYI); *R. rettgeri* O1—O34 (Dr. S. NAMIOKA [17]); *P. inconstans* O1—O61 (Dr. W. H. EWING [16]); *E. coli* O1—O135 (Prof. F. KAUFFMANN, Dr. F. ØRSKOV); *Citrobacter* O1—O32 (Prof. J. SEDLÁK); *Salmonella* O1—O53 (Prof. F. KAUFFMANN).

Preparation of immune sera and antigens was performed by standard methods [1—3]. The homologous agglutinin titre of the sera was in the range 1280—10240.

* Presented in part at the Enterobacteriaceae Symposium, 4th Congress of the Hungarian Association of Microbiologists, Budapest, 1964.

Agglutination and agglutinin absorption. A preliminary slide agglutination was performed with all but *E. coli* strains. Subsequent tube agglutination tests were made first in pooled sera then in monovalent sera [1-3]. Cross-absorption tests were carried out only in case of high titre reciprocal reactions.

Evaluation of results. According to the titre of cross-reaction and the decrease of the homologous antibody level after absorption, three degrees of antigenic relationships were distinguished: "minor", "major" and "identical". When the titre of the cross-reaction was less than 1/10 of that of the homologous strain, the relationship was considered "minor". "Major" relationships were characterized by titres reaching 1/5 to 1/2 of the homologous titre. O antigens were considered identical when in cross-absorption experiments the heterologous antigen removed all antibodies reacting with the homologous strain.

Results

The results are summarized in four tables indicating the reciprocal cross-agglutination titres before and after absorption of the serum, and the character of the relationship.

Table I presents relationships existing between *M. morganii* and *Proteus*. It is seen that antigens *M. morganii* O10 and *P. hauseri* O12 are identical. The identity of antigens *M. morganii* O23 and *P. hauseri* O17 is also evident. An a, b—a, c-type relationship was found between *M. morganii* O5 and *P. hauseri* O38, between *M. morganii* O9 and *P. hauseri* O7 and also between *M. morganii* O11 and *P. hauseri* O33. One relationship was only revealed

Table I

*Antigenic relationships between M. morganii
and other genera of the tribe Proteae*

<i>M. morganii</i>	<i>P. hauseri</i>	Variety of relationship	Degree of relationship
O10	O12	Identical O antigens	
O23	O17	Identical O antigens	
O5	O38	a, b—a, c	minor
O9	O7	a, b—a, c	minor
O11	O33	a, b—a, c	minor
<i>R. rettgeri</i>			
O8	O10	Identical O antigens	
<i>P. inconstans</i>			
O1	O59	a, b—a, c	minor
O2	O60	a, b—a, c	major
O6	O13	a, b—a, c	minor
O10	O13	a —a, b	major

between *Morganella* and *Retzgerella*, antigens *M. morganii* O8 and *R. rettgeri* O10 were namely identical.

With one exception the relationships with *P. inconstans* (*Providencia*) were of the a, b—a, c-type: *M. morganii* O1—*P. inconstans* O59, *M. morganii* O2—*P. inconstans* O60 and *M. morganii* O6—*P. inconstans* O13. An a—a, b-type relationship was found between *M. morganii* O10 and *P. inconstans* O13.

Table II presents the relationships with *E. coli*. The identity of thermostable and thermolabile antigens in *M. morganii* O29 : K1 and *E. coli* O112a, c : K66(B11) was described by RAUSS in 1957 [5]. Recently the antigenic identity of *M. morganii* O34 : K2 and *E. coli* O111 : K58(B4) has been shown [15]. In addition to these the following minor a,b—a,c-type relationships were revealed: *M. morganii* O7—*E. coli* O120, *M. morganii* O10—*E. coli* O29 and O123, *M. morganii* O15—*E. coli* O1, *M. morganii* O20—*E. coli* O62, *M. morganii* O21—*E. coli* O5, *M. morganii* O24—*E. coli* O70 and O122.

Relationships with *Citrobacter* are indicated in the upper part, those with *Salmonella* in the lower part of Table III.

It is seen that an a, b—a, c relationship exists between *M. morganii* O9—*Citrobacter* O14, *M. morganii* O20—*Citrobacter* O29 and *M. morganii* O24—*Citrobacter* O26.

S. invernensis (O38), *S. niarembé* (O44) and *S. bergen* showed an a, b—a, c-type relationship with *M. morganii* O9.

The important finding that *M. morganii* O9 contains O antigenic components present in *P. hauseri* O7, *Citrobacter* O14, *S. invernensis*, *S. niarembé*

Table II
Antigenic relationships between *M. morganii* and *E. coli*

<i>M. morganii</i>	<i>E. coli</i>	Variety of relationship	Degree of relationship
O29 : K1	O112a, c : K66(B11)	Identical O and K antigens	
O34 : K2	O111a, b : K58(B4)	Identical O and K antigens	
O7	O120	a, b—a, c	minor
O10	O29	a, b—a, c	minor
O10	O123	a, b—a, c	minor
O15	O1	a, b—a, c	minor
O20	O62	a, b—a, c	minor
O21	O5	a, b—a, c	minor
O24	O70	a, b—a, c	minor
O24	O122	a, b—a, c	minor

Table III

Antigenic relationships between M. morganii and Citrobacter

<i>M. morganii</i>	<i>Citrobacter</i>	Variety of relationship	Degree of relationship
O9	O14	a, b— a, c	major
O20	O29	a, b— a, c	minor
O24	O26	a, b— a, c	minor

Antigenic relationships between M. morganii and Salmonella

<i>M. morganii</i>	<i>Salmonella</i>	Variety of relationship	Degree of relationship
O9	<i>S. invernness</i> O38	a, b— a, c	major
O9	<i>S. niarembé</i> O44	a, b— a, c	minor
O9	<i>S. bergen</i> O47	a, b— a, c	minor

Table IV

Antigenic relationship between M. morganii O9 and members of other Enterobacteriaceae genera

<i>M. morganii</i> O9	<i>P. hauseri</i> O7	a, b— a, c
	<i>Citrobacter</i> O14	a, b— a, c
	<i>S. invernness</i> O38	a, b— a, c
	<i>S. niarembé</i> O44	a, b— a, c
	<i>S. bergen</i> O47	a, b— a, c

and *S. bergen*, is summarized in Table IV. The O antigen mosaic of the above species, as indicated by cross-agglutination and cross-absorption experiments, can be expressed as follows. *M. morganii* O9, a₁, a₂, b₁, b₂; *P. hauseri* O7, a₂, c; *Citrobacter* O14, b₁, b₂, d; *S. invernness* O38, b₂, e; *S. niarembé* O44, b₂, f; *S. bergen* O47 b₂, g.

It is seen that the related organisms contain complex O antigens, in which the factors responsible for the relationship are themselves composed of different constituents.

Discussion

As it has been pointed out in the introductory part of this paper, studies on antigenic relationships are desirable not only for their use in routine diagnostic work but also in view of theoretical aspects. As to the latter, especially

the chemical analysis of antigens and demonstration of a possible genetic basis of the relationships should be mentioned.

From the present data on the incidence, characteristics and significance of antigenic relationships between *Morganella* and other *Enterobacteriaceae* genera, it seems justified to conclude to the existence of further relationships between these genera.

Most of the revealed relationships are of the a, b—a, c variety, which means that in addition to components responsible for the cross-reaction, the related species contain specific factors. Some a—a, b varieties were also recorded. From our data it is clear that we only dealt with reciprocal relationships and omitted the description of frequently occurring unilateral reactions.

As it follows from the results of reciprocal agglutinations and absorptions, mostly minor relationships were noted; more extensive relationships or even antigenic identities were less frequent.

Antigenic relationships have been the subject of extensive investigations. Systematic studies and examination of single cases are equally numerous. *M. morganii* has, however, been studied less thoroughly. The senior author reported on an antigenic relationship between *Proteus vulgaris* and *M. morganii* as early as 1936 [7]. In 1957 he demonstrated the antigenic identity of a *Morganella* strain and *E. coli* O112a, c : K66(B11) [5]. This *Morganella* strain was later included in the antigenic schema as a representative of serotype O29 : K1 : H19 [3]. RUSTIGIAN and STUART in 1945 demonstrated antigenic relationships between *P. vulgaris*, *P. mirabilis* and *M. morganii*, as well as between *R. rettgeri* and *P. inconstans* [6]. SOMPOLINSKY in 1957 showed an antigenic relationship between *Sh. flexneri* and one of his *P. morganii* O groups [9].

Our studies have presented a systematic study on the relationship between 29 *Morganella* O antigens defined in our schema and different genera of the family *Enterobacteriaceae*.

Of intra-tribe relationships among *Proteae*, the antigenic identity of *M. morganii* O10 and *P. hauseri* O12 as well as of *M. morganii* O23 and *P. hauseri* O17 should be noted. In addition, 3 minor relationships of the a, b—a, c variety were revealed between *M. morganii* and *P. hauseri*. One relationship was only found between *Morganella* and *Rettgerella*: antigens of *M. morganii* O8 and *R. rettgeri* O10 were identical. With *P. inconstans* 3 different a, b—a, c and 1 a—a, b relationships were revealed. In view of the relative rarity of O antigenic identity and minor relationships within *Proteae*, it seems justified to divide the tribe into four different genera. This conception is confirmed by the biochemical properties of the tribe [12].

Among relationships of the O antigens of *Morganella* and *E. coli* the identity of *M. morganii* O29 : K1 and *E. coli* O112a, c : K66(B11) [5] and the

recently revealed identity of *M. morganii* O34 : K2 and *E. coli* O111a, b : K58(B4) [15] antigens are important. In addition, 8 mainly minor, a, b—a, c-type relationships have been shown.

Among the 3 a, b—a, c relationships between *Morganella* and *Citrobacter*, that existing between *M. morganii* O9 and *Citrobacter* O14 was considered important.

One major and 2 minor a, b—a, c relationships were noted between *Morganella* and *Salmonella* O antigens (*M. morganii* O9—*S. invernensis* (O38), *S. niarembé* (O44) and *S. bergén* (O47)).

As regards antigen chemistry, it may be important that the same *Morganella* O antigen is related to certain species of different genera, thus *M. morganii* O9 to *P. hauseri* O7, *Citrobacter* O14, *S. invernensis*, *S. niarembé* and *S. bergén*. It should be noted that the relationship between *S. invernensis* and *Citrobacter* O14 has been described earlier [1, 2]. This finding clearly demonstrated the complexity of antigens of the family *Enterobacteriaceae*. KAUFFMANN has also emphasized that cross-reactions in *Enterobacteriaceae* do not minimize the importance and practicability of serological investigations; on the contrary, their detection and comparison are of considerable practical and theoretical significance [1].

Acknowledgements. The authors are indebted to Professor F. KAUFFMANN (Copenhagen), Dr. F. ØRSKOV (Copenhagen), Dr. W. H. EWING (Atlanta, Ga.), Dr. SH. NAMIOKA (Tokyo), Professor J. SEDLÁK (Prague) and Dr. B. LÁNYI (Budapest), for supplying the cultures. Our thanks are due to Miss E. HRABOVSKY for skilful technical assistance.

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VERGLEICHENDE CHEMISCHE ANALYSE DER LIPOPOLYSACCHARID-ANTIGENE DER SHIGELLA SONNEI-MUTANTEN PHASE I UND II SOWIE R

Von

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(Eingegangen am 18 August 1966)

Zusammenfassung. Es wurden die aus den Mutanten der Phase I (S), der Phase II und R von *Sh. sonnei* hergestellten, serologisch aktiven LPS-e einer vergleichenden chemischen Analyse unterzogen, die feststellen ließ, daß in der Antigenstruktur der drei LPS-e eine große Ähnlichkeit besteht.

Vermutlich wird das Grundgerüst aller drei LPS von einer Polyheptose-Phosphat-Grundkette gebildet, an die Glukose (R), bzw. Glukose und Galaktose (Phase I und II) gekoppelt sind. Die S—R-Mutation betrifft den Galaktoseeinbau bzw. den Galaktosestoffwechsel. Die Ursache für die scharfe serologische Abweichung zwischen dem LPS der Phase I und dem Phase II ist in der feineren Struktur der LPS-Moleküle zu suchen, während in der R-Mutante das Fehlen der Galaktose ihre serologische Charakteristik erklärt. Unsere analytischen Befunde bekräftigen unsere frühere Vermutung, daß die S—R-Mutation der *Sh. sonnei* nach einem weitgehend ähnlichen Mechanismus wie bei *Salmonellen* vor sich geht.

Die Untersuchung der sog. L₁-Fraktionen ließ feststellen, daß sie aus mehreren Komponenten bestehen, die neben Nukleinsäure auch Polysaccharide enthalten und diese aus für das LPS charakteristischen Zuckerkomponenten, sowie Mannose und Fruktose aufgebaut sind.

Die S—R-Mutation der *Enterobacteriaceen* ist auf einen in dem die Lipopolysaccharid (LPS)-Antigensynthese steuernden Enzymsystem eintretenden Defekt zurückzuführen. Untersuchungen mit *Salmonellen* haben erwiesen, daß Mutanten isoliert werden können, die in verschiedenem Grade degradierte LPS-synthetisieren. Die Stufe der Degradation hängt davon ab, an welcher Stelle der Defekt in der Enzymkette eintritt [25].

Seit langem ist bekannt, daß die S—R-Mutation der *Shigella sonnei* zumindest in zwei Phasen abläuft. Die Mutante zwischen S- und R-Form wurde — zur Unterscheidung von der typischen S-Form (Phase I) Phase II genannt [53]. Der Gedanke lag nahe, daß im Falle der *Sh. sonnei* die Degradation der LPS-Synthese in ähnlicher Weise vor sich geht wie bei den *Salmonellen*. Um dies beweisen zu können, mußten zunächst die LPS-Antigene der Mutanten verglichen werden.

In immunchemischer Hinsicht sind die drei Mutanten bisher nur von wenigen untersucht worden. HAAS war der erste, der mittels Trichloressigsäureextraktion aus der Phase I einen Extrakt herstellte [15]. GOEBEL und JESAITIS haben aus Bakterien der Phase I und II Protein-LPS-Antigen hergestellt und dieses chemischen und serologischen Analysen unterworfen [14, 18].

Die neueren Kenntnisse bezüglich der S—R-Mutation ließen es angebracht erscheinen, diese Untersuchungen zu ergänzen und — auch die Mutante R

einbezogen — zusammenzufassen und sich dabei der in der jüngsten Zeit entwickelten immunochemisch-analytischen Methoden zu bedienen.

Material und Methodik

Die Extrakte wurden auf die früher beschriebene Weise [33] von selektierten und gezüchteten Bakterien bereitet. Die Bakterienmasse wurde mit Azeton getrocknet.

Der *Boivin-Extrakt* wurde wie üblich aus 10 g trockenen Bakterien hergestellt [5]. Das erhaltene Protein-LPS-Antigen wurde mittels Ultrazentrifugierung (4 Stunden bei 105 000 g) gereinigt bzw. lyophilisiert.

Extrahierung mit heißem Wasser [18]. 5 g Bakterien (*Sh. sonnei* Phase II und R) wurden in 200 ml dest. Wasser suspendiert und 1 Stunde lang bei 65 °C im Wasserbad erwärmt. Nach scharfem Abschleudern (40 Min. bei 22 000 g) wurde der wasserklare Überstand mit *N* Essigsäure auf pH 3,5–4 gebracht, über Nacht bei +4 °C stehen gelassen, der Niederschlag abzentrifugiert, in 100 ml dest. Wasser suspendiert und das pH mit 0,1 *N* NaOH auf 7,0 eingestellt, gegen dest. Wasser dialysiert und dann lyophilisiert.

Phenol-Wasser-Extraktion [47]. 40 g getrocknete Bakterien wurden in 700 ml Wasser suspendiert und dann mit dem gleichen Volumen 90%igen Phenols auf die übliche Weise bei 65 °C extrahiert. Die Extraktion wurde anstatt 20 Minuten nur 5 Minuten fortgesetzt, und dann der dialysierte und lyophilisierte Extrakt mittels Ultrazentrifugierung (dreimal 4 Stunden 105 000 g) gereinigt. Die Überstände (Fraktion L_1-L_3) und der LPS-Niederschlag wurden lyophilisiert.

(a) Qualitative Bestimmungen

Die qualitative Bestimmung der Zuckerkomponenten mittels Papierchromatographie: 10 mg gefriergetrocknetes Material wurde in 1 ml NH_2SO_4 4 Stunden bei 100 °C hydrolysiert, die Lösung nach Neutralisierung mit $Ba(OH)_2$ eingengt und in einem Gemisch von *n*-Butanol: Pyridin : Wasser 6 : 4 : 3 auf Whatman-1-Papier chromatographiert. Die Entwicklung erfolgte mit Anilinhydrogenphthalat.

Die *Aminosucker* wurden in mit 10 mg 4 *N* HCl 10 Stunden lang hydrolysiertem Material bestimmt, die Hydrolysate zentrifugiert, das Supernatant im Vakuumexsikkator in Gegenwart von NaOH eingetrocknet und der Rest — in Wasser aufgenommen — in dem obigen Laufmittel chromatographiert. Entwicklung: mit Ninhydrin.

Die aus LPS präparierten Heptosen wurden in folgenden Laufmitteln nach DAVIES [6] chromatographiert: Butanol : Pyridin : Wasser 6 : 4 : 3; *n*-Propanol : Benzylalkohol : Wasser : Ameisensäure 50 : 72 : 20 : 20; Aceton : Wasser 95 : 5; und Phenol : Ammoniak. Entwicklung: mit Anilinhydrogenphthalat.

(b) Quantitative Bestimmungen

Die in den Originalvorschriften angeführten Volumina haben wir proportional herabgesetzt, so daß bei den Bestimmungen die photometrierenden Volumina stets ca. 1 ml betrugen. Zu den Bestimmungen dienten Konstriktionspipetten vom Carlsberg-Typ (Fa. H. H. Pederson, Kopenhagen) und Semimikroküvetten (Hellma G. m. b. H., Müllheim/Baden, Typ 104) unter Verwendung des Zeiss PMQ II. bzw. Beckman DU-Spektrophotometers.

Die quantitative Bestimmung der *Hexosen* erfolgte in mit *N* HCl zubereiteten Hydrolysaten. Zur Ermittlung der günstigsten Dauer der Hydrolyse wurden 4–5 mg LPS in *N* HCl im Wasserbad bei 100 °C hydrolysiert und aus den zu verschiedenen Zeitpunkten entnommenen Proben nach PARK und JOHNSON [32] die Reduktionswerte bestimmt. Der maximale reduzierende Wert wurde nach 4–5stündiger Hydrolyse erhalten, doch waren 50% des Gesamtreduktionswertes bereits nach 15–20 Minuten freigesetzt. Dementsprechend wurden 3–4 mg genau gewogenes LPS, bzw. Fraktion L_1 in 400 μ l *N* HCl in zugeschmolzenen Ampullen 4 Stunden bei 100 °C hydrolysiert, der ausgeschiedene Niederschlag wurde abzentrifugiert, vom Überstand 350 μ l vorsichtig abpipettiert und 1 : 1 mit Wasser verdünnt, dann das verdünnte Hydrolysat mit Amberlit IRA 410 $\cdot$ HCO₃-Harz neutralisiert und aus dem Supernatant worden Galaktose-, Glukose-, Mannose-, Ribose- und Fruktosebestimmungen durchgeführt.

Zur Bestimmung der *Aminosucker* wurden 3–4 mg genau gewogenes Material in 400 μ l 4 *N* HCl in zugeschmolzenen Ampullen 10 Stunden bei 100 °C hydrolysiert, der ausgeschiedene Niederschlag wurde abzentrifugiert und aus einem Teil des Überstandes der Glukosamingehalt bestimmt.

Zur *N*- und *O*-Azetylbestimmung wurden 20 mg genau gewogenes LPS in 2,0 ml Wasser gelöst, hiervon 3 $\times$ 300 μ l wurde die Azetylbestimmung vorgenommen; weitere 3 $\times$ 300 μ l

wurden im Vakuum eingetrocknet und dann mit 500 μ l alkalischem Hydroxylamin zwecks Eliminierung der O-Azetylgruppen 3 Minuten bei 65 °C hydrolysiert. Nach dem Zentrifugieren wurde das Sediment dreimal mit je 2 ml Äthanol gewaschen und aus dem Sediment wiederum Azetylbestimmung vorgenommen. Aus der Differenz zwischen den beiden Ergebnissen wurde der Gehalt an O- und N-Azetyl berechnet.

Bei den quantitativen Bestimmungen fanden folgende Methoden Verwendung: *Galaktose*: mit Galaktosedehydrogenase [44] bzw. Galaktoseoxydase [11], *Glukose*: mit Glukoseoxydase (Boehringer u. Söhne, Mannheim), *Mannose*: mit Mannoseisomerase [31], *Fruktose*: mit dem Karbazol-Schwefelsäuretest von DISCHE u. BORENFREUND [7], *Ribose*: nach TRACEY mit Anilinoxalat [43], *Glukosamin*: nach Azetylierung mit Essigsäureanhydrid mit der MORGAN—ELSON-Reaktion nach ROSEMAN u. DAFFNER [36], sowie mit D-Glukosaminazetylase nach LÜDERITZ u. Mitarb. [24], *Heptose*: mit der Zystein-Schwefelsäure-Methode von DISCHE in der Modifikation von OSBORN [29] mit D-Glycero-L-Mannoheptose als Standard, *Keto-Desoxy-Octonsäure* (KDO): mit der Thiobarbitursäure-Methode nach WEISSBACH u. HURWITZ [46] in der Modifikation von HEATH u. CHALAMBOR [17]. — Als Standard diente der Penta-Azetyl-Methylester der KDO, aus dem die KDO nach Verseifung gewonnen wurde [16]. Die *Azetylbestimmung* geschah nach LUDOWIEG und DORFMAN mit Äthylazetat Standard [21]. Der *Estergehalt* wurde nach SNYDER und STEPHENS [39] mit Palmitinsäure-Methylester-Standard ermittelt. *Eiweiß*: nach LOWRY und Mitarb. [20] mit Bovin-Serumalbumin-Standard. Der Gehalt der Extrakte an *Nukleinsäure* wurde auf Grund der bei 260 $m\mu$ gemessenen Extinktion in Beckman DU — oder Cary 14 Registrier-Spektrophotometer festgestellt. Als Standard diente Hefenuklesäure (Merck).

Die *Phosphor-* und *Stickstoffbestimmungen* wurden im Forschungslaboratorium Dr. A. WANDER in Bern ausgeführt.

Der *PS-Gehalt* der Fraktionen der Ionenaustauschchromatographie wurde mit der Phenol-Schwefelsäure-Methode nach DUBOIS und Mitarb. [10] getestet.

Der *Desoxyribonukleinsäure-Gehalt* wurde mit der Diphenylaminmethode nach DISCHE [9] — mit DNS (Reanal) als Standard — geprüft.

Der Fortschritt der *Perjodatoxydation* wurde auf Grund der bei 225 $m\mu$ gemessenen Absorption beurteilt.

Ergebnisse

Herstellung der Antigene. Das Vollantigen von *Sh. sonnei* ist — wie das Körperantigen anderer enteraler Bakterien — ein Protein-LPS-Anteil die serologische Spezifität trägt. Zur Bereitung dieses Vollantigens ist das gebräuchlichste Verfahren die Trichloressigsäure-Methode von BOIVIN und MESROBEANU [5], doch kann mit ihr lediglich aus der Phase I das Protein-LPS-Antigen hergestellt werden [33], so daß wir aus den Bakterien der Phase II bzw. der Mutante R das Vollantigen mit der von JESAITIS und GOEBEL angewandten Heißwasser-Methode herstellten [18]. Diese Extrakte wurden aber nur zur Bestimmung des Eiweißanteiles verwendet (s. Tabelle III). Zu den übrigen analytischen Untersuchungen dienten die mit der Phenol-Wasser-Methode hergestellten, durch Ultrazentrifugation gereinigten LPS-e.

Die mittels Ultrazentrifugierung sedimentierten LPS-e wurden lyophilisiert, ebenso auch der nach der ersten Ultrazentrifugierung erhaltene Überstand, die sog. Fraktion L₁, die neben Nukleinsäure auch Polysaccharide enthielt (Tabelle I).

Das gereinigte LPS der Phase I ist in Wasser leicht löslich, während das der Phase II und R nur mittels Erwärmens eine homogene Suspension gibt, deren Stabilität gering ist, und die schon nach Zentrifugieren mit niedriger Umdrehungszahl (2000 g) sedimentiert. Aus diesem Grunde konnte die analy-

Tabelle I*Ausbeute der LPS- und L₁-Fraktionen aus je 40g Bakterienzellen*

Sh. sonnei	LPS g	%	L ₁ g	%
Phase I	0,605	1,5	3,400	8,5
Phase II	0,940	2,3	1,418	3,7
R	0,875	2,2	1,240	3,1

tische Ultrazentrifugation der beiden letzteren Stoffe nicht verwirklicht werden. Das LPS der Phase I erscheint in der analytischen Ultrazentrifuge als ein breites »Peak«.

Die gereinigten LPS-Fraktionen waren auf Grund des Absorptionsspektrums frei von Nukleinsäuren.

Vergleich der LPS- und L₁-Fraktionen. Um zu entscheiden, ob ein Unterschied in der Kohlehydratzusammensetzung des nach Ultrazentrifugation erhaltenen Sediments (LPS) und des Überstandes — (L₁-Fraktionen) — besteht, wurden papierchromatographische Untersuchungen herangezogen und dabei das gereinigte LPS und die Fraktion L₁ benutzt (Tabelle II).

Zwischen dem LPS und den L₁-Fraktionen sind qualitative Unterschiede festzustellen; der auffallendste darunter ist das Fehlen der Mannose im LPS und ihre Anwesenheit in der Fraktion L₁. Galaktose findet sich nur im LPS der Phasen I und II, und in Spuren in der L₁-Fraktion der Phase I.

Im Gegensatz zu den LPS-en enthalten die L₁ Fraktionen stets Mannose. Zu bemerken ist, daß in dem angewandten Laufmittel auch die Fruktose mit gleichen Rf-Wert wie die Mannose läuft. LÜDERITZ und Mitarb. [22] beobachteten ein häufiges Vorkommen der Fruktose in den L₁-Fraktionen der Salmonellen, was aus den obigen Gründen Anlaß zur Verwechslung mit der Mannose gibt. An den mit Anilinphosphat entwickelten Chromatogrammen hatte der Mannosefleck oft einen rötlichen Stich, was auf das Vorhandensein von

Tabelle II*Zuckerzusammensetzung der LPS-e und der L₁-Fraktionen*

	Phase I		Phase II		R	
	LPS	L ₁	LPS	L ₁	LPS	L ₁
Galaktose	+	±	+	—	—	—
Glukose	+	+	+	+	+	+
Mannose—Fruktose	—	+	—	+	—	+
Glukosamin	+	+	+	+	+	+
Heptose	+	±	+	±	+	+
KDO	+	—	+	—	+	—
Ribose	—	+	—	+	—	+

Pentose bzw. Ketozucker hindeutet. Die mit Mannoseisomerase vorgenommene Auswertung hat die Anwesenheit sowohl von Mannose, als auch von Fruktose erwiesen.

Quantitativ-chemische Analyse der LPS-Fractionen (Tabelle III). Aus den Chromatogrammen geht hervor, daß das LPS der Phasen I und II aus vier Zuckern: Galaktose, Glukose, Heptose und Glukosamin aufgebaut ist. Aus dem R—LPS fehlt die Galaktose. Die LPS-e enthalten die obigen Kohlenhydrate in nicht reduzierbarer Form. Bei der Hydrolyse mit normal Mineralsäuren bei 100 °C wird rund die Hälfte des Gesamtreduktionswertes bereits nach 15—20 Minuten freigesetzt. Das Lipoid A wird zu dieser Zeit noch nicht vollkommen abgespalten: es ist auch mit heißem Chloroform nicht auszusütteln, hierzu ist eine halbstündige Hydrolyse erforderlich. Nach 30 Minuten Hydrolyse konnten wir — im Gegensatz zu den bei LPS-en anderer Herkunft gemachten Erfahrungen [37, 42] — neben großen Mengen Monosacchariden nur verschwindend kleine Mengen Oligosaccharide nachweisen. Für die Säurehydrolyse erwies sich besonders das LPS der Phase I als empfindlich.

Sehr aufschlußreich ist das Studium des Verhältnisses der einzelnen Kohlenhydratkomponenten. Die auffallendste Veränderung zeigt — wie auch nach den papierchromatographischen Ergebnissen zu erwarten war — die Verringerung des Galaktosegehaltes. Die Glukose bleibt praktisch unverändert. Der Heptose- und Phosphorgehalt nimmt parallel mit dem Deutlicherwerden des R-Charakters zu: dies weist darauf hin, daß die Polyheptose-Phosphat-Grundkette einen immer größeren Teil des Polysaccharids bildet [30]. In

Tabelle III
Quantitative Zusammensetzung der LPS-Fractionen

	Phase I	Phase II	R
Galaktose	7,2	6,5	—
Glukose	6,2	9,8	8,0
Glukosamin	7,5	9,8	10,5
Heptose	13,5	18,4	18,9
KDO	6,9	9,6	14,8
N-azetyl	3,3	0,7	0,3
O-azetyl	0,7	0,5	0,7
Stickstoff	4,2	2,2	1,8
Phosphor	2,5	4,0	3,6
Ester	12,1	14,2	15,4
Protein	30,5*	85,7**	83,6**

* im Boivin-Extrakt

** im Wasser-Extrakt

gleicher Weise nimmt auch der KDO-Gehalt zu. Das molare Heptose : Phosphat : KDO-Verhältnis beträgt bei allen drei LPS annähernd 2 : 1 : 1.

Zum Heptosenachweis bedienen wir uns der Zystein-Schwefelsäuremethode von DISCHE, weil die in den *Sh. sonnei* vorkommende Aldoheptose in dem benutzten papierchromatographischen Laufmittel keinen separaten Fleck gibt, sondern zwischen dem Galaktose- und Glukosefleck Platz nimmt und dadurch gleichermaßen die Grenzen dieser beiden Flecken verwischt [6]. In dem

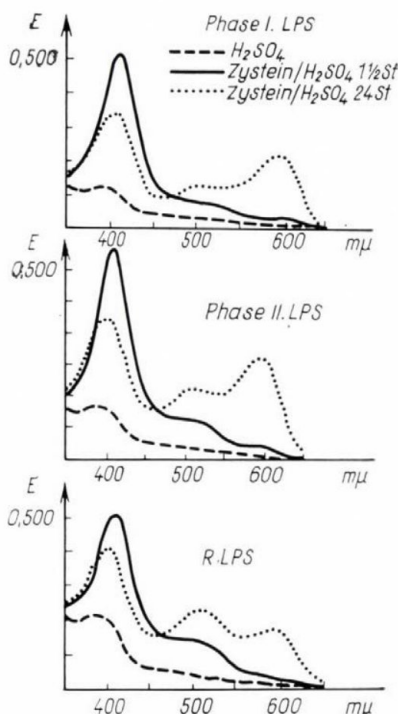


Abb. 1. Spektren der DISCHE-Reaktion der LPS-e

sekundären Spektrum der sekundären Zystein-Schwefelsäure-Reaktion nach DISCHE jedoch deutet das bei ca. 510 $m\mu$ erscheinende Absorptionmaximum auf das Vorhandensein von Aldoheptose hin (Abb. 1).

Wie auch die sekundäre DISCHE-Reaktion zeigt, nimmt der Heptosegehalt von der Phase I in Richtung des R-Typ stetig zu.

Zwecks näherer Identifizierung haben wir die aus den drei LPS-e stammenden Heptosen nach SLEIN und SNELL [38] präpariert: 20 mg LPS wurden in 1 ml Wasser gelöst, mit 1 ml 2 N HCl versetzt und 3 Stunden bei 100 °C hydrolysiert. Durch Zugabe von 1 ml 2 N NaOH wurde das pH auf 8,0–9,9 eingestellt und dann dem Hydrolysat 50 mg festes Bariumazetat beigelegt. Die trübe Lösung wurde zentrifugiert, der Überstand über Nacht bei 0 °C

mit 3 Volumen 96%igem Äthanol gefällt, nach dem Zentrifugieren das Sediment — welches das Bariumsalz des Heptosephosphats enthielt — mit Wasser extrahiert, durch Zugabe von 2 *N* H₂SO₄ zu den Überständen das Barium ausgefällt, das Supernatant in zugeschmolzenen Ampullen 16 Stunden bei 100 °C hydrolysiert und nach anschließender Neutralisierung chromatographiert. Als Ergebnis der chromatographischen Untersuchungen konnte festgestellt werden, daß die aus allen drei LPS stammende Heptose an unseren Chromatogrammen entsprechend der D-Glycero-L-Mannoheptose, oder L-Glycero-D-Mannoheptose lief. Dieses Ergebnis deckt sich mit der Feststellung von GOEBEL und JESAITIS [14], die die im LPS der Phase II gefundene Heptose mit der D-Glycero-L-Mannoheptose identifizierten.

Nun wurden die LPS-e nach FOSTER und Mitarb. mit Perjodat behandelt. Diese Autoren hatten nämlich festgestellt, daß wenn man das LPS-Molekül mit Natrium Perjodat oxydiert und dann reduziert — unter günstigen Umständen aus der in das Polysaccharid eingebauten Heptose die entsprechende Hexose — so aus der L-Glycero-D-Mannoheptose D-Mannose — entsteht [13].

Nachdem die *Sh. sonnei*-LPS-e Mannose nicht enthalten, war dieses Verfahren anwendbar. An den Chromatogrammen der Perjodatoxydierten (und dann mit Natrium borhydrid reduzierten) LPS-Hydrolysate erschien in allen drei Fällen ein Mannose-Fleck, der für das Enzym D-Mannosedehydrogenase ein geeignetes Substrat bildete. Somit kann als erwiesen gelten, daß die *Sh. sonnei*-LPS-e L-Glycero-D-Mannoheptose enthalten.

Glukosamin und Galaktosamin zeigen in dem benutzten Laufgemisch einen gleichen Rf-Wert, deshalb haben wir zur Identifizierung des Aminozuckers Ninhydrin-Abbau nach STOFFYN und JEANLOZ [41] vorgenommen. An den Chromatogrammen wurde nur ein der Arabinose entsprechender Fleck gefunden, das heißt, die LPS-e haben höchstwahrscheinlich nur Glukosamin enthalten. Bekräftigt wird diese Vermutung durch den Umstand, daß die bei der spezifischen Bestimmung mit enzymatischen Methoden [24] und die mit der Morgan-Elson-Reaktion erhaltenen Ergebnisse [36] — innerhalb der zulässigen Fehlergrenze — übereinstimmen.

Das Glukosamin ist ein Bestandteil des Lipoid A [52], kann aber auch am Aufbau des LPS teilnehmen [25]. Werden die LPS-e etwa 30 Minuten bei 100 °C mit *N* Mineralsäure erwärmt, so wird das Lipoid A abgespalten und ausgefällt [49]. Um entscheiden zu können, ob das Glukosamin im Aufbau des LPS teilnimmt oder aber nur im Lipoid A vorkommt, wurde je rund 10 mg LPS in 1 ml *N* HCl 30 Minuten bei 100 °C hydrolysiert. Während der Hydrolyse schied aus dem LPS der Phase II und des R das Lipoid A in Form eines flockigen Niederschlages aus, der sich selbst sedimentierte; beim LPS der Phase I war der Niederschlag nur zum Teil sedimentiert, der andere Teil war suspendiert geblieben und konnte nur durch Zentrifugieren (ca. 10 Minuten bei

2000 g) abgeschleudert werden. Nach dem Kühlen wurde mit 2 ml Chloroform geschüttelt und zentrifugiert, die wässrige Phase vorsichtig abgesaugt, die chloroformhaltige Phase mit 1 ml *N* HCl ausgeschüttelt und zentrifugiert. Die wässrigen Phasen wurden vereint und sowohl die wässrige, als auch die Chloroformphase in Vakuum eingetrocknet. Der Rückstand wurde mit je 1 ml 4 *N* HCl 10 Stunden bei 100 °C hydrolysiert und daraus die Glukosaminbestimmung vorgenommen (Tabelle IV).

Tabelle IV

Die Verteilung des Glukosamins zwischen PS und Lipoid A

	% Glukosamin in	
	PS	Lipoid A
Phase I	28,0	70,0
Phase II	3,0	95,0
R	0,3	99,1

Die Hauptmasse des Glukosamins fanden wir im Lipoid A. Der im PS-Anteil der Phase I gefundene, relativ hohe Glukosamingehalt kann möglicherweise das Ergebnis der ungenügenden Hydrolyse sein, doch ist trotz alledem nicht auszuschließen, daß das Glukosamin auch im Aufbau des Polysaccharids der Phase I beteiligt ist.

Der Stickstoffgehalt ist teils dem Glukosamin, teils den an die LPS-e gebundenen Peptiden zuzuschreiben. Die Abnahme des Stickstoffes in der R-Mutante mit immer stärkerem R-Charakter (Phase II → R) demonstriert die ständig nachlassende Fähigkeit des LPS-Moleküls, Eiweiß zu binden.

Hinsichtlich des N-Azetylgehaltes weicht das LPS der Phase I scharf von jenem der Phase II und der R-Mutante ab; im O-Azetylgehalt ist ein größerer Unterschied zwischen den drei Mutanten nicht zu beobachten; das gleiche gilt auch für den Estergehalt.

Die Perjodat-Oxydation der LPS-e. In Vorversuchen wurden ca. 5 mg LPS in 500 µl Wasser gelöst, dem bei +4 °C 100 µl 0,1 *N* Perjodsäure zugesetzt wurde. Zeitweise entnommene Proben von 25 µl wurden mit 5 ml dest. Wasser verdünnt und ihr Perjodatverbrauch wurde gemessen. Die erhaltene Kurve zeigte daß dieser in 2 Stunden praktisch vollendet ist.

Nun wurden in 2,5 ml Wasser 25–30 mg gereinigtes LPS gelöst nach Abkühlen auf +4 °C mit 0,5 ml 0,1 *N* Perjodsäure versetzt und nach Ablauf von 2 Stunden wurde mit 0,25 ml Äthylenglykol das restliche Perjodat zersetzt. Nach 15 Minuten folgte Reduktion des perjodatbehandelten LPS 15 Minuten bei Raumtemperatur mit 10 ml 2%igem Natriumborhydrid und anschließend Zersetzen des restlichen Borhydrids mit 5 ml Eisessig, dann wurde

das Reaktionsgemisch 3 Tage gegen dest. Wasser dialysiert und gefriergetrocknet.

Die Perjodatoxydation ging bei allen drei LPS-en mit annähernd gleicher Geschwindigkeit vor sich (Abb. 2).

Von dem behandelten Material wurden rund je 70 bzw. 35% (R LPS) zurückgewonnen. Das Ergebnis der Analysen der perjodat-oxydierten LPS-e zeigt Tabelle V.

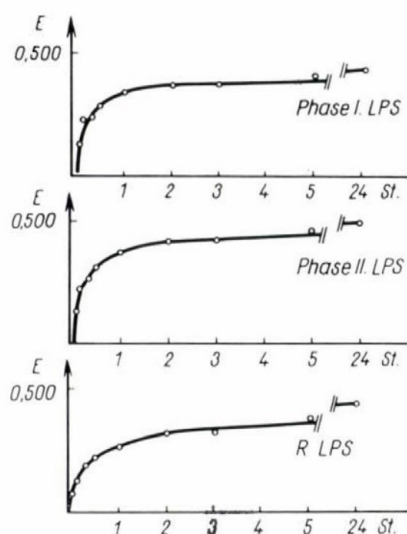


Abb. 2. Perjodat-Verbrauch der LPS-e

Im Laufe der Perjodatoxydierung war die Galaktose vollkommen zer-
setzt, der Glukosegehalt hatte sich bei den Phasen I und II praktisch nicht
verändert und im Falle von R um rund 25% herabgesetzt, während der
Glukosamingehalt sich beim LPS der Phasen I und II um rund 25%, und
beim R um ca. 30% vermindert hatte.

Tabelle V

Quantitative Analyse der Zuckerkomponenten der perjodatoxydierten LPS-e

	Phase I %	Phase II %	R %
Galaktose	—	—	—
Glukose	6,5	9,8	6,0
Mannose	3,8	5,3	4,7
Glukosamin	5,6	7,3	7,3
Heptose	+	+	+

Wie bereits weiter oben erwähnt, entsteht im Laufe des Verfahrens aus Heptose Mannose. Das Ergebnis der quantitativen Mannosebestimmung aber weist darauf hin, daß im Laufe der Perjodat-oxydation nur aus etwa einem Drittel der Gesamtheptose Mannose entsteht.

Auch die mit perjodat-oxidierten, LPS-en aufgenommenen DISCHE-Spektren zeigen, daß die LPS-e noch beträchtliche Menge unzersetzte Heptose enthalten (Abb 3).

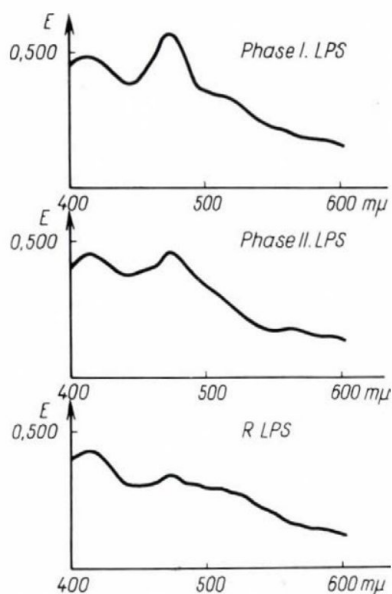


Abb. 3. Spektren der sekundären DISCHE-Reaktion der mit Perjodat oxydierten LPS-e

Eine genaue quantitative Bestimmung ist allerdings nicht durchführbar, weil im Laufe der Perjodatoxydation und der darauffolgenden Reduktion ein Produkt mit einem Maximum zwischen 530 und 540 $m\mu$ entstand, welches das in der DISCHE-Reaktion bei 510 $m\mu$ erscheinende Absorptionsmaximum der Heptose stark überdeckt.

Analyse der L_1 -Fraktionen. Die Analyse der L_1 -Fraktionen erfolgte mit den bei den LPS-en benutzten Methoden, ergänzt mit Bestimmungen, welche durch die Natur des Materials angezeigt erschienen. Die Ergebnisse sind in Tabelle VI enthalten.

Die L_1 -Fraktionen enthalten nur sehr winzige Mengen (L)PS ihre Hauptmasse besteht aus Ribonukleinsäure. Dies beweisen das in den UV-Spektren bei 260 $m\mu$ gefundene Absorptionsmaximum und der Ribosegehalt. Es scheint, daß die L_1 -Fraktion der *Sh. sonnei*-Mutanten auch für das LPS charakteristische Komponenten enthält: in unseren Substanzen fanden wir Glukosamin,

Tabelle VI
Quantitative Analyse der L₁-Fraktionen

	Phase I %	Phase II %	R %
Galaktose	0,2	—	—
Glukose	0,9	1,8	0,8
Mannose	2,5	0,8	1,7
Fruktose	1,5	0,7	0,8
Glukosamin	2,1	2,7	4,5
Heptose	5,5	2,5	5,5
Protein	3,3	3,7	6,2
RNS	40	53	30
DNS	11	8	17

und die DISCHE-Spektrogramme lassen auf das Vorhandensein von Heptose schließen (Abb. 4).

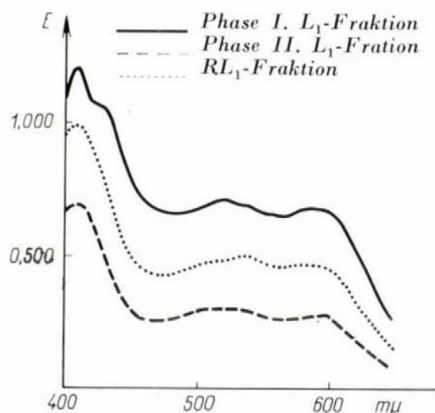


Abb. 4. Spektren der sekundären DISCHE-Reaktion der L₁-Fraktionen

Der Mannosegehalt wurde aus der Differenz zwischen den mit und ohne Mannoseisomerase erhaltenen Keto-hexosewerten errechnet. Den Fruktosegehalt erbrachte die direkte Keto-zuckerbestimmung.

In der analytischen Ultrazentrifuge verhalten sich die L₁-Fraktionen als polydispers [3]. Zur Trennung der einzelnen Komponenten bedienten wir uns der Säulenchromatographie.

Mit DEAE-Sephadex A—50 Ionenaustauscher-Dextrangel (Pharmacia, Uppsala, Sweden), das zuvor nach den vorgeschriebenen Rezyklisierungen mit M/15 Phosphatpuffer vom pH 7,2 äquilibriert worden war, wurde eine Säule von 27×290 mm hergestellt und auf diese 500 mg in 5 ml Phosphatpuffer

gelöste L_1 -Fraktion gebracht. Die Eluierung wurde mit 500 ml Puffer eingeleitet und dann mit Gradient-Elution — durch Hinzufließenlassen von 500 ml M NaCl in $M/15$ Phosphatpuffer zu 1000 ml Puffer — fortgesetzt. Die Fraktionen wurden in je 6,5 ml Portionen gesammelt und hinsichtlich ihres Nukleinsäure- und Polysaccharidgehaltes untersucht. Die erhaltenen Chromatogramme sind in Abbildung 5 dargestellt.

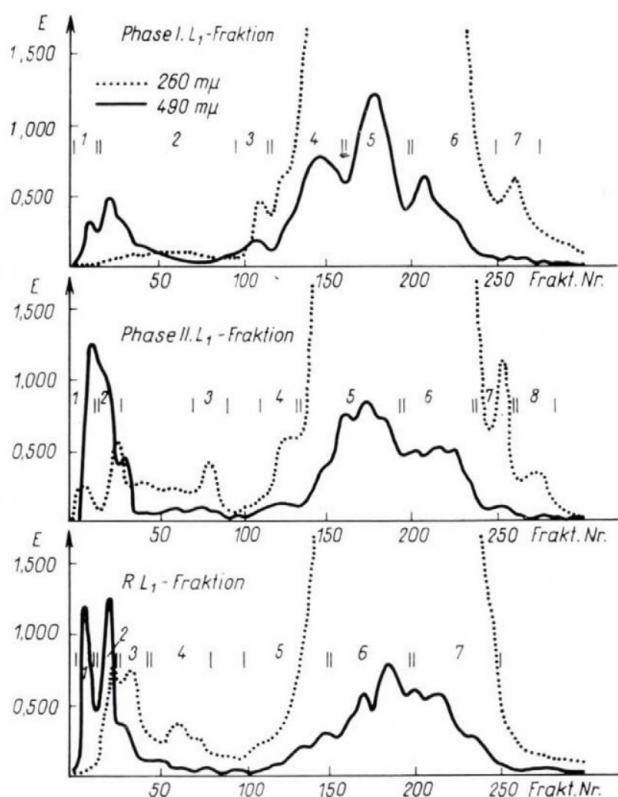


Abb. 5. Chromatographie der L_1 -Fraktionen an der DEAE-Sephadex-Säule

Die den einzelnen Maxima entsprechenden Fraktionen wurden gesammelt, gegen dest. Wasser dialysiert und lyophilisiert. Die Ergebnisse der Analyse der so gewonnenen L_1 -Komponenten zeigt Tabelle VII.

Die Chromatogramme der L_1 -Fraktionen der drei Mutanten weisen Ähnlichkeiten auf, sie sind sämtlich aus mehreren Komponenten zusammengesetzt. Von der Säule löst sich zunächst eine unbeladene (L)PS-Gruppe fast mit dem »void volume« ab [12]. Diese enthält keine oder nur geringe Menge im UV-Bereich absorbierender Substanz.

Der mit Hilfe der Gradienten-Elution ablösbare, aus mehreren Komponenten bestehende Nukleinsäureanteil enthält im Falle der Phase I und

Tabelle VII

Zusammensetzung der Komponenten der an der DEAE-Sephadex-Säule getrennten L_1 -Fraktionen

Komponenten Nr.	Phase I							Phase II								R						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7
Galaktose	—	—	—	S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Glukose	+	+	—	+	+	+	—	+	+	—	—	—	—	—	—	+	+	+	+	—	S	—
Mannose bzw. Fruktose	+	+	—	—	—	—	—	+	+	—	—	+	—	—	—	+	+	—	—	—	—	—
Glukosamin	—	—	+	+	+	S	—	—	—	—	+	+	—	—	S	—	+	+	+	+	+	—
Heptose	+	+	—	+	—	—	—	+	+	—	—	—	—	—	—	+	+	—	—	—	—	—

S = Spuren

R auch Glukose und Galaktosamin. In der Nukleinsäure ist in allen drei Fällen neben RNS auch DNS enthalten.

Besprechung

Die O-Antigeneigenschaften der Enterobakterien werden von dem in der Zellwand befindlichen LPS-Eiweißkomplex getragen, und die serologische Spezifität ist durch die feinere chemische Struktur des Polysaccharidanteiles dieses Komplexes bestimmt. In den letzten Jahren hat sich — vorwiegend als Ergebnis der Arbeiten von WESTPHAL, LÜDERITZ, KAUFFMANN, OSBORN, NIKAIDO [51, 25, 19, 29, 27, 28] und anderen die Ansicht entwickelt, daß diese hochspezifische Struktur auf ein relativ aspezifisches Grundgerüst aufgebaut ist, welches aus der an das sog. Lipoid A gekoppelten Polyheptose-Phosphat-kette besteht. An dieses Grundgerüst sind dann unter Vermittlung der Glukose die weiteren Monosaccharid-Bausteine gekoppelt, die letzten Endes mittels mannigfacher Variationen der chemischen Bindungen dem Molekül seine hohe Spezifität verleihen.

Diese Hypothese, die tagtäglich durch immer neuere Beweise bekräftigt wird, beruht hauptsächlich auf den Ergebnissen der Versuche, die an Salmonellen durchgeführt worden sind. Besonders lehrreich war das Studium der S—R-Mutanten. Die Bakterien können nämlich durch Mutation ihre Fähigkeit zur Bildung eines in der Synthese des O-Antigens teilnehmenden Enzyms verlieren, und so kann die Antigensynthese — wegen Fehlens des betreffenden Enzyms — auf einer gegebenen Stufe stehenbleiben, wodurch anstatt des vollständigen Moleküls nur halb aufgebaute, über andere Spezifitäten verfügende Moleküle zustandekommen können, aus denen meistens — in Abhängigkeit von der Stelle des Enzymdefektes — auch irgendeine für die S-Form (normale Variante) charakteristische Kohlenhydratkomponente fehlt. Man konnte bei

Salmonellen R-Mutanten finden, die hinsichtlich der Zusammensetzung ihrer LPS-e in eine Reihe eingestuft werden konnten. Die LPS-e mit einfacherem Aufbau repräsentieren die unteren Stufen der Synthese. Es ist z.B. nachgewiesen worden, daß das eine der einfachst gebauten LPS-e als einzige Zuckerkomponente des PS-Anteiles nur Heptose enthält [25].

Ob die Antigensynthese einer anderen bedeutenden Gruppe der Enterobakterien, der Shigellen, nach einem dem oben beschriebenen Mechanismus ähnlich abläuft, ist nur von wenigen untersucht worden.

Sh. sonnei sind für derartige Untersuchungen gut geeignet, da — wie seit langem bekannt — ihre S—R-Mutation in mehreren, auch serologisch gut erfaßbaren, Phasen vor sich geht.

Wir haben unsere Arbeit mit der serologischen Untersuchung der drei Mutanten begonnen und konnten feststellen, daß während die Phase I über die typischen S-Eigenschaften verfügt, die Phase II und R immer deutlicher die klassischen R-Eigenschaften aufweisen [33].

Ziel der vorliegenden Arbeit war zu untersuchen, welcherart chemische Unterschiede zwischen den drei Mutanten nachweisbar sind und in welcher Beziehung sie zu den serologischen Eigenschaften stehen.

Schon die Extrahierbarkeit der Antigene zeigte große Unterschiede. Während aus den Bakterien der Phase I das LPS-Eiweiß-Antigen mit dem Boivinschen Trichloressigsäure-Verfahren extrahiert werden konnte, führte diese Methode im Falle der Bakterien der Phase II und R nicht zum Ziel. Wie wir in früheren Arbeiten darauf hinwiesen [33], ist die Ursache hierfür in dem Verhältnis Eiweiß: LPS innerhalb des Molekülkomplexes zu suchen. Die analytischen Befunde haben diese Vermutung bekräftigt: Der Antigenkomplex der Phase II und der Mutante R (Eiweiß-LPS) enthält etwa über mehr als das Zweieinhalbfache an Eiweiß als das Vollantigen der Phase I.

Auch die physikalischen Eigenschaften der mit Hilfe des Phenol-Wasser-Verfahrens gewonnene LPS-e erwiesen sich als verschieden: die Wasserlöslichkeit der LPS-e von Phase II und R läßt in Richtung R progressiv nach; dies deutet auf eine verminderte Hydrophilität der PS-Komponente hin, deren Ursache die verminderte Hydratation des über kürzere Seitenketten verfügenden Moleküls sein dürfte [51].

Betreffs der Zuckerkomponenten besteht zwischen den LPS-en der Phasen I und II kein Unterschied. Die in der Antigenstruktur eingetretene Veränderung ist vermutlich auf die Veränderung des Bindungstyps zurückzuführen, die sich wahrscheinlich beim Einbau der Galaktose geltend macht. Hierfür spricht die Verringerung der relativen Galaktosemenge. Die bisher erhobenen Befunde liefern keinen eindeutigen Beweis, doch ist anzunehmen, daß bei den Bakterien der Phase II — bei unverändert intensiver Galaktose-synthese — der durch das galaktoseübertragende Enzym gebildete Bindungstyp verändert ist. Im Falle der R-Mutante muß das Fehlen des galaktose-

einbauenden Enzyms — bzw. eine Störung der Galaktosesynthese — als eine weitere Stufe der Veränderung in Richtung R angesehen werden.

In Analogie zu der bei den Salmonellen beobachteten, bereits erwähnten Synthesedegradation [25] ist anzunehmen, daß auch *Sh. sonnei*-R-Mutanten isoliert werden können, die als weiteren Grad der Degradation einen Defekt in ihrem Glukose- bzw. Heptosestoffwechsel erleiden. GOEBEL und JESAITIS [14] haben mit Hilfe der Phagen-Technik aus *Sh. sonnei*-Bakterienkulturen der Phase II phagenresistente Kolonien isolieren können, in deren LPS-Antigen die für die Phase II typischen Zucker (Galaktose, Glukose, Heptose und Glukosamin) fehlten. Mit der gleichen Technik konnte unlängst eine R-phagenresistente Mutante isoliert werden können, in deren LPS — nach den vorläufigen Untersuchungen — nur Heptose nachweisbar ist [1].

Das Verhältnis der Heptose und Glukose erfährt in den drei Mutanten keine wesentliche Änderung, was auf die Beständigkeit der Grundstruktur hinweist. Die Veränderung des KDO-Gehaltes geht der des Heptosegehaltes parallel. Dies stimmt mit den Beobachtungen von HEATH [17] und OSBORN [29, 30] überein, wonach die KDO im Aufbau der Grundstruktur des Polysaccharides teilnimmt.

Das Vorkommen natürlicher Aldoheptose haben als erste JESAITIS und GOEBEL gerade bei *Sh. sonnei* beobachtet [18]. Auf Grund ihrer Stelle auf dem Chromatogramm vermuten sie, da es sich hier um die D-Gala-L-Mannoheptose (D-Glycero-L-Mannoheptose) handelt. WEIDEL [45] hatte aus *E. coli*-LPS Aldoheptose isoliert und festgestellt, daß diese mit der L-Glycero-D-Mannoheptose identisch ist. Auf Grund der relativ engen Verwandtschaft zwischen *Sh. sonnei* und *E. coli* nahm er an, daß auch in *Sh. sonnei* Heptose mit L-Konfiguration vorkommt. Unsere experimentellen Befunde bekräftigen die Feststellungen dieses Autors: die aus mit Perjodat oxydiertem LPS stammende Mannose wird durch die d-Mannose-Isomerase umgewandelt, und so ist es sehr wahrscheinlich, daß in allen drei *Sh. sonnei*-Mutanten tatsächlich L-Glycero-D-Mannoheptose vorkommt.

Der relativ hohe Stickstoffgehalt des LPS der Phase I legt die Vermutung nahe, daß das stark verzweigende Molekül gebundene Eiweißanteile bzw. Peptide enthält, welche die Phenolbehandlung nicht vollkommen beseitigen konnte. Die Eiweißbindungsfähigkeit des LPS der einfacheren, weniger verzweigten gebauten Mutante der Phase II bzw. R ist geringer.

Das Glukosamin dürfte in N-azetylierter Form vorkommen. Im LPS der Phase I wurde ein auffallend hoher N-Azetylgehalt beobachtet, die gefundene Menge betrug etwa das Doppelte des zur Azetylierung des gesamten Glukosamins nötigen Wertes.

Es ist wahrscheinlich, daß das Glukosamin nur am Aufbau des Lipoid A beteiligt ist [49, 51]. LÜDERITZ und Mitarb. [25] haben beschrieben, daß das Glukosamin auch an der Bildung der serologischen Spezifität teilnimmt. Nach

Abspalten des Lipoid A haben wir auch in der Polysaccharidfraktion geringe Mengen von Glukosamin gefunden, doch ist mit der benutzten Methode [52] das Lipoid A nicht vollkommen abspaltbar: nach Entfernung des Lipoid A schied bei weiter fortgesetzter Hydrolyse noch immer eine lipoidartige Substanz aus.

In der Perjodat-Oxydation zeigte das LPS der Phasen I und II keine wesentliche Abweichung. Es ist vorstellbar, daß die Galaktose in beiden LPS-en terminal lokalisiert ist und sich nur in ihrer Bindungsweise an die Glukose unterscheidet. Dies ist verständlich, da ja minimale Änderungen in der terminalen Struktur des PS erhebliche Abänderungen in der serologischen Spezifität verursachen können.

Die Glukose blieb im Falle des LPS der Phasen I und II intakt, während sie im LPS der R-Mutante während der Perjodat-Oxydation bis zu rund 25% abgebaut wurde. Auch dies spricht dafür, daß die Glukose im LPS der Phasen I und II wahrscheinlich nicht terminal angeordnet ist.

Das Glukosamin scheint in einer mit Perjodat oxydablen und einer perjodat-resistenten Bindungsform zugegen zu sein. Das gleiche gilt auch für die Heptose, von der nur rund ein Drittel Mannose lieferte. Im DISCHE-Spektrum des perjodat-oxydierten LPS ist das bei 510 $m\mu$ erscheinende Maximum, welches die Anwesenheit der nach der Perjodatoxydation unversehrt gebliebenen Heptose anzeigt, noch deutlich erkennbar.

Die L_1 -Fraktionen wurden zuerst von BECKMANN und Mitarb. [3, 4] untersucht. Die Autoren hatten festgestellt, daß in der L_1 -Fraktion gewisser *S. typhi murium* R-Stämme ein für die S-Form charakteristisches, nicht zu LPS komplettiertes, serologisch aktives PS vorkommt. Im Falle der *Sh. sonnei* ist besonders interessant, daß wir in der L_1 -Fraktion sogar zwei Zucker — Mannose und Fruktose — fanden, die im Aufbau der LPS-e nicht teilnehmen (abgesehen von der Ribose, die vermutlich ein Bestandteil der RNS-Komponente ist). Gleichzeitig wurde in den L_1 -Fraktionen Galaktose nur in geringer Mengen oder überhaupt nicht gefunden, während sie einen Bestandteil des LPS der Phasen I und II bildet. Gegenüber anderen Autoren können wir die Beobachtung, wonach in der L_1 -Fraktion die für das Lipoid A und das LPS charakteristische Komponenten, wie Glukosamin und Heptose, nicht enthalten seien, nicht bekräftigen. Diese wurden in den einzelnen Fraktionen nicht in dem für das LPS typischen Verhältnis gefunden, so daß die Vermutung, ihre Anwesenheit sei eine Folge der Verunreinigung der L_1 -Fraktionen, fallen gelassen werden muß.

Es fragte sich, ob die Mannose bzw. Fruktose wohl ein Homopolymer bilden oder aber in Form eines mit anderen Zuckerkomponenten zusammen gebildeten PS vorhanden sind. Unsere Untersuchungen haben gezeigt, daß diese Zucker zusammen mit den in der L_1 -Fraktion gefundenen anderen Zuckerkomponenten ein Molekül bilden: dies aber beeinflußt — im Vergleich zum

LPS — die serologische Spezifität der L_1 -Fraktion nicht. Das gegen das ganze Bakterium gerichtete Immunserum wird auch von dem Mannose und Fruktose nicht enthaltenden LPS vollkommen absorbiert. Eine Ausnahme bilden die Bakterien von R-Typ, die nach den Untersuchungen von RAUSS und KONTROHR [34] über ein vom LPS unabhängiges, fest an die Zellwand verankertes Antigen verfügen. Dieses Antigen ist aber in der L_1 -Fraktion nicht nachweisbar und wird durch Phenolbehandlung zerstört.

Heptose haben wir in den L_1 -Fraktionen nur in Geringer Menge gefunden. Erschwert war ihr Nachweis dadurch, daß in der sekundären DISCHE-Reaktion neben der Heptose die über ein Maximum bei 530—540 $m\mu$ verfügende Ribose — aus der RNS — und eine nicht identifizierte, ein Absorptionmaximum bei 470 $m\mu$ gebende Substanz — wahrscheinlich die DNS-Desoxyribose — das bei 510 $m\mu$ erscheinende, schwache Heptose-Maximum verdrängt.

Mit der zum KDO-Nachweis benutzten Thiobarbitursäure-Methode erhielten wir bei den die Hauptmasse der Ribonukleinsäure vertretenden Fraktionen eine positive Reaktion. Diese Farbreaktion ist vermutlich nicht der KDO, oder eventuell in der L_1 -Fraktion vorhandenen anderen Stoffen — wie z.B. Sialsäure — zuzusprechen, sondern ist durch die neben der RNS vorhandene DNS bedingt.

Der größte Teil der Komponenten der L_1 -Fraktion stammt aus dem Zellplasma [3, 4]. Ihre Analyse breitet ein sehr wechselvolles Bild vor uns aus. Zieht man in Betracht, daß in den meisten isolierten Fraktionen für das LPS typische Komponenten gefunden wurden, so ist leicht vorstellbar, daß diese Stoffe Fragmente der LPS-Synthese sind, die ein der nächsten Stufe der Synthese die für den Organismen so charakteristischen Makromoleküle zustandebringen.

Für die freundliche Überlassung der synthetischen 2,4,5,7,8-Pentaazetyl-methyl-KDO-Probe entbieten die Verfasser Herrn Dr. E. C. HEATH (John Hopkins University, Baltimore, Md.) und für die authentische D-Glycero-L-Mannoheptose Herrn Dr. N. K. RICHTMYER (National Institutes of Health, Bethesda, MD.) auch an dieser Stelle ihren aufrichtigen Dank. Für die P- und N-Bestimmungen sind wir dem Berner Forschungslaboratorium Dr. A. WANDER zu Dank verpflichtet.

Der eine der Verfasser (T. K.) möchte der Max-Planck-Gesellschaft für das ihm zuerkannte Stipendium auch hier seinen verbindlichen Dank aussprechen.

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CLINICAL AND SEROLOGICAL OBSERVATIONS ON AUTOGENOUS AND HETEROGENOUS VACCINATION IN STAPHYLOCOCCAL SKIN DISEASES

By

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(Received September 19, 1966)

Summary. Autovaccine, Smith diffuse and compact vaccine and commercial polyvalent vaccine were given to 49 patients suffering from chronic and relapsing staphylococcal skin diseases. Good therapeutic results have been obtained with auto and SMITH's diffuse vaccine, but not with SMITH's compact and commercial polyvalent vaccine. The favourable clinical response was accompanied by a rise in phagocytic index and mouse protective antibodies. No relationship was demonstrated between immunity and the titre of direct and indirect (Coombs) haemagglutinins. Alpha antitoxin levels showed no significant alterations and remained under the normal values.

Immunity developing in staphylococcal infection is antitoxic and antibacterial. The first experiments on this subject were those of RICHET and HÉRICOURT [1], who inoculated dogs with staphylococci and examined the protective effect of the animals' sera in rabbits. Subsequent investigations failed to elucidate whether the immunity was strain, type or group specific, in other words, they yielded no data as to the distribution among various strains of the genus of the factor responsible for specific immunity. FARREL and KITCHING [2] and BRODIE *et al.* [3] claimed that immunity was type specific. In contrast, GRÜN and ARNOLD [4], who carried out their studies on the basis of OEDING's antigenic schema, demonstrated that immunity was not type specific. In animal experiments ANGYAL [5] obtained data that confirmed the opinion of GRÜN and ARNOLD, and showed that immunization was successful not only with bacterial cells but also with extracts. In spite of the large number of examinations and the widespread clinical application of vaccines [6, 7], little is yet known about antistaphylococcal immunity. From the studies of MORSE [8] and LI, KAPRAL and MUDD [9] it may be concluded to opsonin-type immunity. It is not yet clear, however, whether vaccination produces protective antibodies or only serologically demonstrable antibodies [10, 11].

The purpose of the present investigations was (1) to observe the effect of vaccination; (2) to compare in the light of data obtained in animals, the immunogenicity in humans of SMITH's diffuse and compact vaccine; and (3) to examine the properties of sera of vaccinated individuals.

Materials and methods

Vaccination was performed with autovaccine, heterovaccine and a commercial polyvalent vaccine. Auto- and heterovaccines were prepared in our laboratory by suspending 18 to 20 hour agar slant cultures of coagulase positive *Staphylococcus aureus* strains in physiological saline containing 0.3 per cent phenol. The suspension was heated at 60° C for 1 hour and adjusted to contain 500 million cells per ml. Heterovaccines were produced from diffuse and compact variants of the Smith strain. All vaccines were tested for sterility and toxicity.

Vaccination was performed intracutaneously with a starting dose of 0.1 ml, then with gradually increasing doses at 3 to 4 day intervals. In most cases 5 doses were given, several patients received 1 or 2 further doses. The patients were tested for sterility and toxicity.

The patients were miners treated as surgical outpatients. They suffered from staphylococcal skin infections lasting for several months or even years with frequent relapses leading to disability to work.

Blood samples were taken before and after vaccination. The sera were stored at -20° C. Except for phagocytic index determination [9], the sera were inactivated at 56° C.

Strains. Autovaccination and serological typing were performed with strains cultured from the patients. Strain Smith was kindly supplied by Dr. I. Joó (Institute for Serobacteriological Production and Research Human, Budapest). The compact variant was isolated by use of the medium described in reference [12]. The two variants were identical in mouse virulence with the strains supplied by Dr. D. E. ROGERS (Vanderbilt University, Nashville, Tenn, U. S. A.).

Type determination was performed as described in the studies of HAUKENES and OEDING [27-32], with the following antigens: a_1 , a_3a_4 , a_5 , b_1 , c_1 , e , f , h_1 , i_1 , i_2 , b_1k , k_1 , m , n . Antigen f was determined with serum 3189 absorbed by strains 2095 and 1015 [13].

Haemagglutination. Direct and indirect (Coombs) haemagglutinins were demonstrated by TAKÁTSY's microtechnique [14]. Sheep erythrocytes were sensitized with GRASSET's antigen [15] at 37° C for 1 hour, then washed twice before use. Non-agglutinating erythrocytes were washed twice in phosphate buffer and tested with Coombs serum (rabbit antihuman gamma globulin) diluted 1:10.

Alpha antitoxin titre was determined at the 1:100 level as described by SZATHMÁRY [16] with alpha toxin prepared from the Wood 46 strain and with standard alpha antitoxin (Human, Budapest).

Phagocytic index was determined according to the method of ROGERS and MELLY [17]. Leukocytes were obtained from one donor. The suspension was washed and stored in Hanks' solution containing 1:10,000 heparin, 0.5 per cent inactivated rabbit serum and 100 mg-per 100 ml of glucose. The test was carried out in a system containing 10 per cent of the examined serum. The bacterial cell: leukocyte ratio was 2:1. The Smith diffuse variant was employed as test strain.

Immunoelectrophoresis. The patient sera were run for 1 hour in the LKB (Type 6800 A) apparatus at 250 V. Antihuman horse serum batch 249 was used for precipitation.

Passive mouse protection test was performed with pooled sera. Appropriate serum dilutions were given to mice subcutaneously 1 hour before challenge. The animals were infected intraperitoneally with the Smith diffuse variant suspended in 5 per cent mucin. Readings were made after 24 hours.

Statistical analysis. The ED_{50} value was estimated as described by KÄRBER [18].

Results

Clinical observations. Around the site of intracutaneous vaccination a hyperaemic reaction 0.5 to 1 cm in diameter was seen for some days. The vaccines caused no other local or general reaction. Results are summarized in Table I.

Autovaccine was given to 13 patients 2 of whom suffered from relapsing hydradenitis and 11 from relapsing furunculosis. Recovery was complete in 12 patients; recurrences were not observed except in one patient after a 2 month latency period.

Table I
Effectiveness of vaccination

Vaccine	Number of patients		
	Recovered	Not recovered	Total
Autovaccine	12	1	13
Smith diffuse vaccine	15	—	15
Smith compact vaccine	2	10	12
Commercial polyvalent vaccine	3	6	9

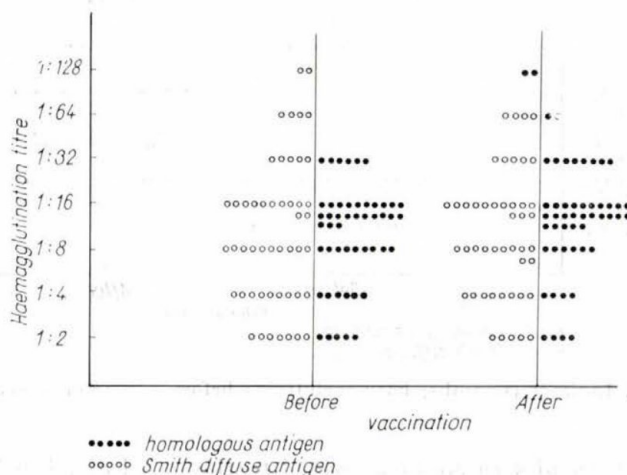


Fig. 1. Direct haemagglutinins before and after vaccination

Vaccine prepared from the *Smith diffuse variant* was given to 15 patients (2 with relapsing hydradenitis and 13 with relapsing furunculosis). The effect was remarkably favourable: recurrence was observed in 1 patient after 7 months. Thus the diffuse vaccine seemed equivalent to autovaccines in therapeutic and preventive effect.

Vaccine prepared from the *Smith compact variant* was used in 12 patients. In 7 patients no beneficial effect was observed, in 5 patients recovery was slow. In 4 of these cases recurrences occurred in 2 to 3 months. It has, therefore, been concluded that the compact vaccine was practically ineffective.

Commercial vaccine was administered to 9 patients with relapsing furunculosis. Only 3 were cured, the rest had relapses.

Examination of haemagglutinins. All sera were examined by direct and indirect (Coombs) haemagglutinins against the homologous culture and Smith diffuse strain.

Fig. 1 indicates that the homologous and Smith strain yielded identical direct haemagglutination titres. Serum samples taken before vaccination showed titres not higher than 1 : 128. The titre of most specimens was 1 : 8 and 1 : 16. Rise in titre after vaccination was observed only in 6 patients, but the titres were still lower than normal.

The titre of indirect (Coombs) haemagglutinins was similar for the homologous and Smith antigens (Fig. 2). A slight rise occurred after vaccination, but no relationship was demonstrated between the effectiveness of vaccination and the indirect haemagglutination titre.

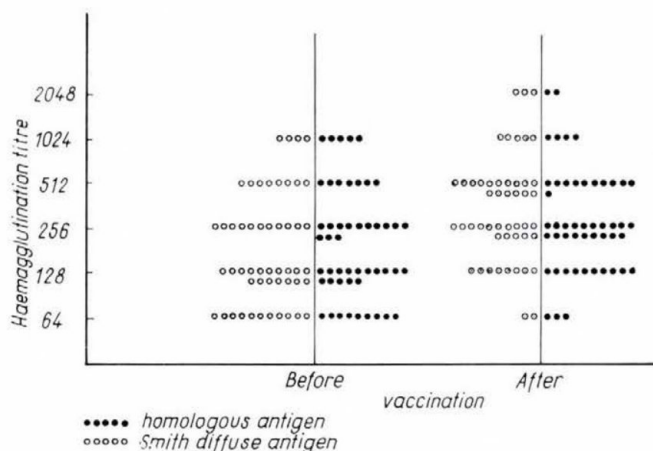


Fig. 2. Indirect (Coombs) haemagglutinins before and after vaccination

The incidence of serotypes conformed to the usual distribution of staphylococcal types (Table II). Type $a_3a_4b_1kc_1$ was the most common. Other strains contained partial antigens in many different combinations. Serotype distribution of strains from patients treated with Smith diffuse vaccine showed no association between the favourable effect of this vaccine and the serotype of the causative agent. This finding confirmed the observations that immunity is not type-specific.

Alpha antitoxin titre of paired sera (Table III) was determined mainly in order to show whether successful vaccination was or was not associated with an elevated alpha antitoxin content. Some authors [4, 19] claimed that vaccination causes no increase in these antibodies, while others [20] stated that even a minimal rise was significant. We observed elevated titres only in 3 patients, one of whom had been injected with autovaccine, the others with commercial vaccine. In the others the rise did not exceed the normal level of 2.5 I.U. High alpha antitoxin levels were found in 3 patients before vaccination; the titres remained unaltered after vaccination. The average titre before vaccination was 1.7 I.U.

Table II

Staphylococcal serotypes isolated from patients treated with different vaccines

Autovaccine	Smith diffuse vaccine	Smith compact vaccine	Polyvalent vaccine
a ₁ if	b ₁	a ₁ b ₁	a ₁ a ₃ a ₄ e
a ₃ a ₄ m	b ₁ kc ₁	ih ₁ mn	a ₁ fmn
a ₁ fmn	ni	emn	a ₅ n
fm	a ₁ ifmn	a ₃ a ₄ h ₁	a ₃ a ₄ m
b ₁ c ₁	a ₃ a ₄	a ₃ a ₄ b ₁	a ₁ ef
fmn	ifn	a ₃ a ₄ b ₁ k	a ₁ b ₁ h
a ₃ a ₄ b ₁ kc ₁	b ₁ kc ₁ ifn	a ₃ a ₄ eh ₁	b ₁
a ₃ a ₄ b ₁ kc ₁	a ₃ a ₄ b ₁ kc ₁	b ₁	a ₃ a ₄ b ₁ kc ₁
a ₃ a ₄ b ₁ kc ₁	a ₃ a ₄ h ₁	b ₁	a ₃ a ₄ b ₁ kc ₁
b ₁ kifmn	a ₃ a ₄ h ₁	a ₁ b ₁ h ₁	
a ₃ a ₄ e	b ₁ kc ₁	a ₃ a ₄ b ₁ kc ₁	
a ₁ b ₁	a ₃ a ₄ b ₁ kc ₁	inagglutinable	
ni	a ₃ a ₄ b ₁ kc ₁		
	inagglutinable		
	inagglutinable		

The phagocytic index (Table IV) is a fairly good indicator of the effectiveness of vaccination. It is seen that, for all patient groups, the number of cells exerting phagocytosis increased after immunization by about 40 per cent in the first 15 minutes of testing. On prolonged incubation this proportion increased further for sera of patients treated with autovaccine and Smith diffuse vaccine. With sera of patients who had been treated with Smith compact vaccine or commercial vaccine, the number of phagocytosing cells showed a decreasing tendency on prolonged incubation.

Table III

Alpha antitoxin titre of sera before and after vaccination

I.U.	Autovaccine		Smith diffuse vaccine		Smith compact vaccine		Polyvalent vaccine	
	B	A	B	A	B	A	B	A
0—1	4	3	6	6	5	6	4	2
1—2.5	7	8	9	9	7	6	4	6
2.5—10	2	2	—	—	—	—	1	1

B = before vaccination;
A = after vaccination.

Table IV

Phagocytic index of sera taken before and after vaccination

Per cent phagocytg cells	Before vac- cination		Autovaccine			After vaccination						Polyvalent vaccine		
						Smith diffuse vaccine			Smith compact vaccine					
	30'	60'	15'	30'	60'	15'	30'	60'	15'	30'	60'	15'	30'	60'
0—10	35													
10—20	11	3												
20—30	3	8												
30—40		37										4		
40—50		1	8			7			9			3	4	3
50—60			4	7		5	2		2	10	2	2	4	1
60—70			1	5	9	2	11		1	2	9		1	5
70—80				1	4	1	2	15						

Passive mouse protection test was performed with pooled sera. Table V shows the ED_{50} for different serum pools of the patients' groups. Autovaccines and Smith diffuse vaccine exerted the highest protective activity: the corresponding serum pools were effective at 1.5×10^{-4} dilution against a 20 LD_{50} challenging dose of the Smith diffuse strain. No significant rise in protective effect was demonstrated in the serum of patients treated with compact and commercial vaccine.

Table V

Passive mouse protection test with sera taken before and after vaccination
(Challenging dose, Smith diffuse variant 20 LD_{50})

Before vaccination	ED_{50} values of pooled sera			
	Autovaccine	Smith diffuse vaccine	Smith compact vaccine	Polyvalent vaccine
10^{-2}	1.5×10^{-4}	1.5×10^{-4}	10^{-2}	2×10^{-2}

Immune-electrophoresis. The sera were subjected to immune-electrophoretic analysis with special regard to gamma globulin, β_2M and β_2A fractions. The patient sera showed no difference from normal sera.

Discussion

A long experience with autovaccines has shown their favourable effect in chronic, relapsing staphylococcal infection. Our studies, in addition to having confirmed this observation, have demonstrated that a heterologous

vaccine prepared from the Smith diffuse variant is as effective as the auto-vaccine. Our results are in agreement with studies demonstrating in animal experiments the high virulence and good immunogenicity of this organism. It may, therefore, be supposed that the diffuse variant contains the protective antigen which produces immunity to infections caused by a wide variety of staphylococcal serotypes. Thus, most *Staphylococcus* strains seem to contain this antigen. The failure of numerous vaccinations [33] may perhaps be attributed to the use of a compact variant or to some inadequate method of vaccine preparation which results in a low immunogenicity of the protective antigen. This consideration is supported by the observation that success is ensured only by preparations made under defined conditions [6, 7]. In view of these observations, our finding that extracts prepared from the Smith strain induce a good immune response [5] seems to have some importance. This type of vaccine may be useful for the immunization of humans.

Serological testing of antibacterial immunity is difficult. The phagocytic index and the passive mouse protection test seem to be associated with clinical results. The former indicates the opsonic character of staphylococcal immunity and is suitable for the demonstration of immunity. Haemagglutinin titre of sera against staphylococcal antigens is not specific and may be attributed to an antigen common in Gram positive bacteria [21]. As serologically distinct strains cannot be distinguished by haemagglutination, it has been supposed that a common factor is responsible for this reaction [22]. Among the available pertaining papers there is only one to state that vaccination had no effect on the haemagglutinin titre of human serum [17]. Our experiments have supported the correctness of this observation and shown that haemagglutinins present in the 7S fraction of serum [23] are unsuitable for the demonstration of immunity. Some authors [24] have shown higher haemagglutinin titres in normal sera than those we found in the present study and which are similar to those of ROGERS and MELLY [17]. The discrepancies are obviously due to differences in the methods.

Determination of indirect (Coombs) antibodies, although these are considered more sensitive indicators [25], also failed to give valuable results. During immunization the titres showed an insignificant rise and were not associated with the clinical picture. As it had been expected, no increase occurred in the alpha antitoxin titre. It should be noted that even vaccines containing toxoid may fail to induce a definite rise in alpha antitoxin titre [33]. Opinions differ as to the average level of alpha antitoxin; for the present material the data of DÓBIÁS *et al.* [26] have been accepted.

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Printed in Hungary

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An Official Journal of the British Society for Immunology

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Vol. 12. No. 6

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IN VITRO ANTIGEN FIXING CAPACITY OF ORGANS FROM IMMUNIZED RABBITS

By

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(Received October 18, 1966)

Summary. Cell-bound antibodies in tissues have been examined by comparing specific and aspecific antigen fixation in the presence of increasing amounts of antigen. Repeated washings were found to decrease the aspecifically bound antigens, while they had no influence on immune-specific binding. Increasing the doses of antigen, the amount of antigen bound by tissue antibodies increased until a maximum had been attained. The method was found suitable for the approximative quantitative estimation of tissue-bound antibodies.

The importance of tissue-bound antibodies has become generally accepted. The two most feasible methods for the determination of tissue-bound antibodies are the passive cellular transfer and antibody titration *in vitro* by determining the specific antigen binding capacity in organ homogenates of sensitized or immunized animals. The latter method has, however, the disadvantage that homogenates may also adsorb antigens in an aspecific way [1].

The degree of aspecific adsorption is different with the various proteins, for instance albumins are less readily adsorbed than globulins [2, 3]. The aspecific adsorption of albumins was decreased [2, 4] by adding small amount ($2 \mu\text{g N}/10^8$ cells) to lymph node or spleen cells of untreated guinea pigs. After an appropriate incubation period and three repeated washings, albumin was found to have been adsorbed very slightly if at all. In sensitized guinea pigs the specific fixation of albumin could convincingly be demonstrated by this type of experiment.

We have shown [1] that blood-free organ homogenates from rabbits immunized with ovalbumin were fixing the antigen in similar amounts, both specifically and aspecifically, in the presence of a larger amount of ovalbumin.

Attempts have, therefore, been made to determine the *in vitro* ovalbumin-fixing capacity of homogenates from rabbits, untreated or immunized with ovalbumin. In these experiments the amounts of ovalbumin added, as well as the number of washings were varied in order to reduce aspecific adsorption to a minimum, without changing the specific antigen-antibody reaction. The principles of the quantitative precipitation method were applied in these studies.

Materials and methods

Antigen. Three times recrystallized ovalbumin (OA) and its ^{51}Cr -labelled chromovalbumin derivative (^{51}Cr -OA) were prepared in this laboratory [5]. The labelled protein was filtered through a Sephadex G-200 column and the pure OA fraction was used.

Preparation of tissue homogenates. 17 rabbits of both sexes, 2500–3000 g in weight, were exsanguinated for studying aspecific adsorption. One gramme (wet weight) samples of liver and lung were homogenized in 2 ml distilled water by means of a Potter apparatus. The enzymes in the homogenate were inactivated by heating to 56°C for 30 minutes.

Thirteen rabbits were hyperimmunized. The animals received 120 mg OA at 4-day intervals, intravenously on the first two occasions and then intraperitoneally, until a total amount of 1–1.2 g of antigen had been administered. The animals were exsanguinated on the 5th–8th day following the last injection. Liver and lung homogenates from the animals were prepared as described above.

Determination of the protein-binding capacity of homogenates. All homogenates were adjusted to pH 6.9 to 7.1, by Tris (tris-hydroxymethyl-aminomethane) buffer. Increasing amounts of ^{51}Cr -OA (10, 20, 30, 40, 50 μg N) were added to samples of the homogenates and the volume was made up to 4 ml with saline. The mixtures were incubated for 50 minutes at 37°C , then cooled to $+4^\circ\text{C}$ and centrifuged for 20 minutes at 10,000 r. p. m. The precipitate was washed 3 times in cold saline.

Radioactivity assay. The activity of supernatants, washing fluids and washed homogenates was assayed by means of a NaI (Ti) Bohrloch crystal scintillation counter. The measured activities were referred to a ^{51}Cr -OA standard of specified N content. The amount of antigen labelled by ^{51}Cr was expressed in terms of N content.

Quantitative precipitation test [6]. This method was used for the determination of antibody content in the sera from rabbits immunized with OA and the amount of antigen required for the adsorption of antibody in the region of equivalence.

Haemoglobin assay. Washing the organs free of blood being known to remove part of the tissue-bound antibody, this method was omitted and a correction factor was used by determining the organs' blood content by haemoglobin assay. The method of TÓTH and JÓKAY [8] was used to determine the haemoglobin content of 1 g samples of each organ. The maximum amount of antigen bound by the blood in the sample was calculated by the equation,

$$x = \frac{a \cdot b}{10} \cdot \frac{E_0}{E_s}$$

where E_0 represents the extinction proportional with the organ's Hgb content; E_s the extinction of haemoglobin in 0.1 ml blood; (a) the plasma volume of 1 ml blood; and (b) the amount of antigen required for precipitating the antibody content of 1 ml serum (in the region of equivalence).

Results

The amount of ^{51}Cr -OA adsorbed by the organ homogenates obtained from the 17 untreated rabbits was determined. Results and deviations are shown in Fig. 1.

The amount of bound protein increased parallel to that of protein added to homogenates of lungs or livers. The amount of aspecifically bound protein amounted to 14 to 20 per cent. To decrease this value, the homogenates were washed. The first washing caused a decrease of about 50 per cent. After 3 washings, aspecific binding was reduced to 2 to 4 per cent.

To each lung and liver homogenate from the 13 immunized rabbits 20 μg N ^{51}Cr -OA was added. The amount of antigen bound by the homogenate without washing, or after 1, 2 and 3 washings, was determined. Mean values and deviations obtained are shown in Fig. 2.

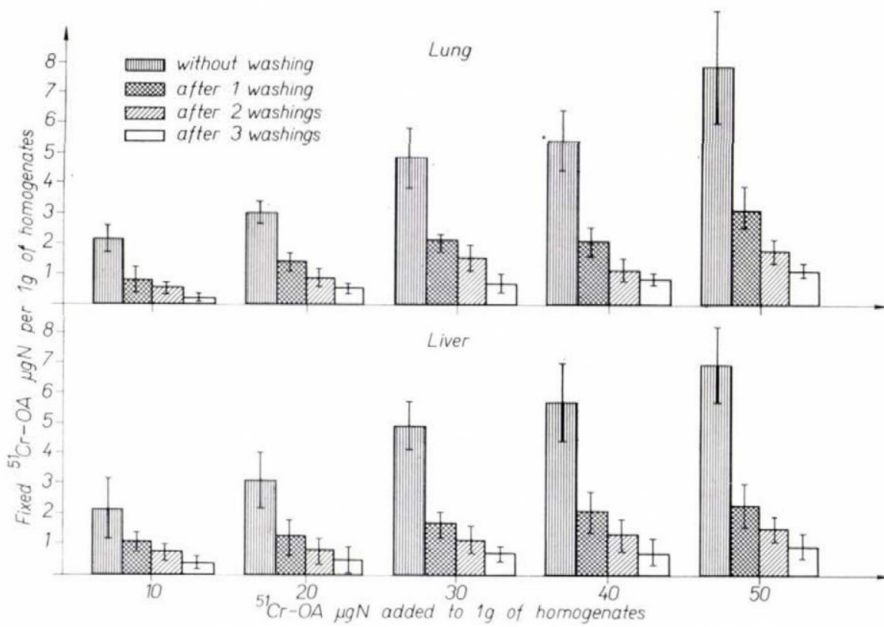


Fig. 1. Binding of $^{51}\text{Cr-OA}$ by organs of control rabbits

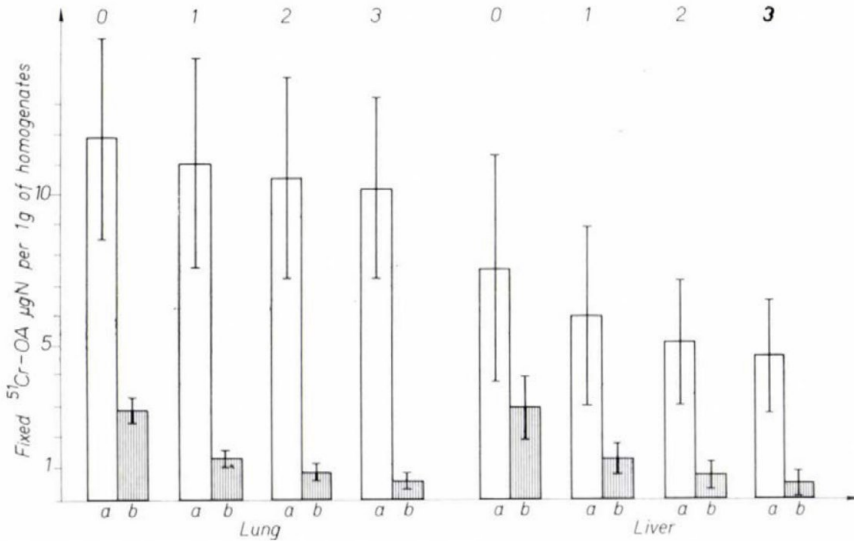


Fig. 2. Effect of repeated washing on binding of $^{51}\text{Cr-OA}$ by organ homogenates from immunized (a) and control (b) rabbits

Columns designated with 0: without washing

Columns designated with 1: after 1 washing

Columns designated with 2: after 2 washings

Columns designated with 3: after 3 washings

To the organ homogenates 20 μg N containing $^{51}\text{Cr-OA}$ was added

The relatively high deviations found in immunized animals may be explained by the variability of the antibody-producing capacity of the individual animals. For comparison, the aspecific antigen fixations observed with organ homogenates from untreated rabbits are also shown. It appeared that repeated washing reduced only the amount of aspecifically bound antigens.

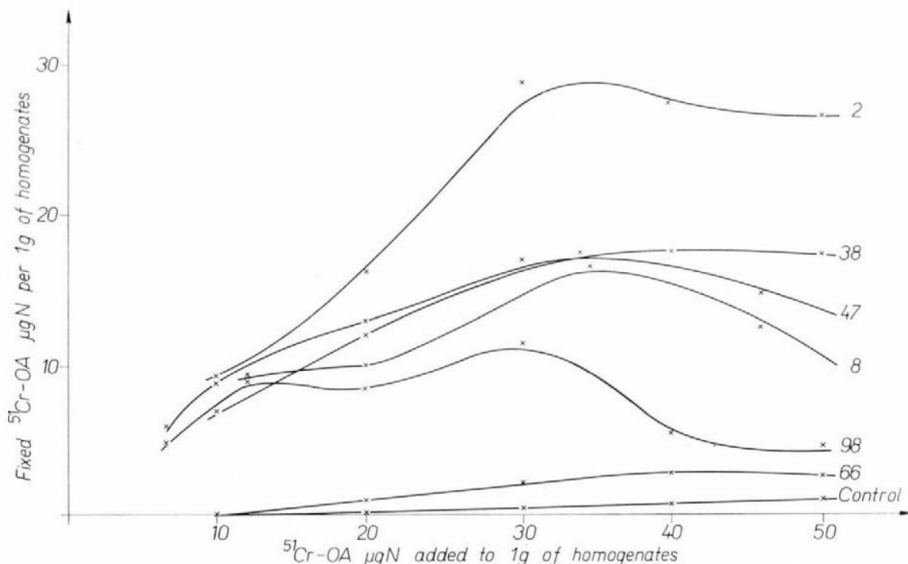


Fig. 3. Binding of ^{51}Cr -OA by lungs of immunized rabbits after 3 washings
The figures designate the serial numbers of the rabbits

The amount of specifically bound antigen, however, remained unchanged even after repeated washing. Comparison of the data obtained in immunized and untreated rabbits suggested that the amount of antigen fixed by lung homogenates from immunized animals contained about 33 per cent of aspecifically bound antigen. After 3 washings, however, the amount of aspecifically fixed antigen decreased by 1/15th. Unwashed liver homogenates from immunized rabbits exhibited about 50 per cent aspecific antigen binding; after 3 washings, this was reduced to 1/9th.

Increasing the concentration of OA also had an influence on its fixation by lung (Fig. 3.) and liver (Fig. 4.) homogenates from immunized animals.

With the increase of the ^{51}Cr -OA concentration, the quantity of fixed antigen did not rise so evenly as in the untreated animals (see results after 3 washings), instead a maximum curve specific for each animal was observed. Accordingly, instead of mean values, some individual results are presented. Thus the amount of antigen bound by organ homogenates from immunized rabbits represented three different bindings, *viz.* aspecific protein adsorption,

specific antigen binding by the blood remnants, and by the tissue antibodies. The first could be obtained from the aspecific binding of untreated animals' organs. From the haemoglobin content in the organs and the amount of antigen needed to bind the serum antibodies, the antigen binding by blood antibodies existing in the organ could be computed. The peaks of curves in Figs. 3 and 4

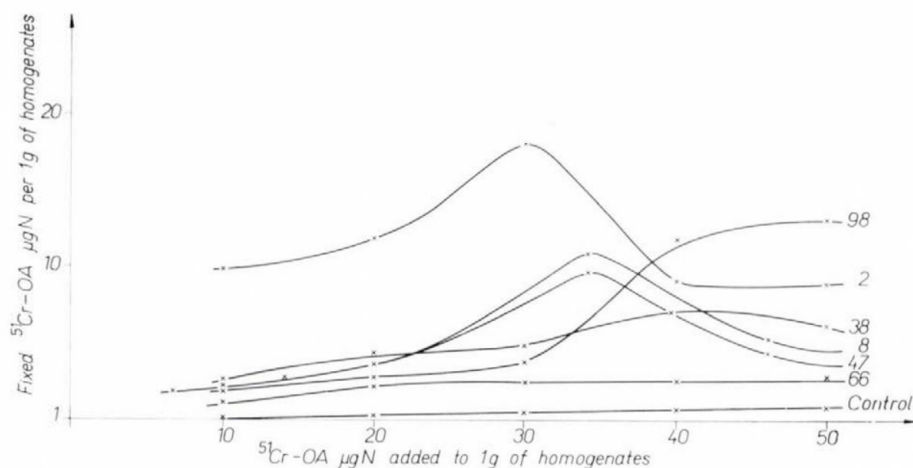


Fig. 4. Binding of ^{51}Cr -OA by livers of immunized rabbits after 3 washings
The figures designate the serial numbers of rabbits

Table I

No. of rabbits	Serum antibody level ($\mu\text{g N/ml}$)	Maximum antigen binding ($\mu\text{g N/ml}$)		
		by serum	by liver homogenate*	by lung homogenate*
70	850	98.6	1.1	6.2
8	1100	99	6.1	9.4
47	1060	99	6.9	11.7
2	1570	159.8	6.2	15.2
38	820	78.4	4.7	11.8
72	380	39.2	0.08	0.0
98	600	59.8	11.8	8.0
12	980	98.6	2.2	1.5
66	180	19.6	2.2	1.9
71	320	32.4	0.1	1.8
23	90	9.8	0.9	0.9

* The amount of antigen bound by the controls and by the serum present in the sample has been subtracted.

represent the maximal amount of antigen bound by the organ as well as by the blood antibodies. Subtracting the latter from the total, we obtained the amount of antigen specifically bound by the homogenate.

In Table I we present the amount of circulating antibody in the serum, the maximum amount of antigen bound by it as well as by the homogenates of liver and lung samples.

From these data it is clear that the amount of antibody bound by the homogenates of the liver and lung is not negligible and that there is no correlation between the amount of circulating and fixed antibodies.

Discussion

The protein binding capacity *in vitro* of animal cells and tissues has been studied extensively. NIELSEN [9], studying the IgG binding capacity of guinea pig ileum *in vitro*, has shown the binding to be an adsorption in the physical sense following the laws by LANGMUIR and being partly removable by repeated washing. The IgG binding capacity of guinea pig lung was examined *in vitro* by BROCKLEHURST *et al.* [10], in relation to time, temperature, protein concentration and number of washings. We have shown [1] that OA was adsorbed more intensively by homogenates than by intact cells and adsorption was found to increase parallel to the concentration of protein added. The present experiments have shown that non-specific adsorption by organ homogenates could be reduced to a minimum by washing.

It can reasonably be supposed that organ homogenates from immunized rabbits exert some aspecific antigen adsorption. This, however, could be reduced by repeated washing similarly to that observed in non-immunized animals. The amount of specifically bound antigen did not seem to be affected by repeated washing. The error caused by the amount of aspecifically bound antigen after three washings is so low that its influence on the determined amount of specifically bound antigen is negligible.

STEFFEN and ROSAK [11] have shown that increasing the amount of added antigen results in an increase of the amount of antigen bound by the lymph node cells. We have also found that increasing the amount of added OA resulted in an increase of the amount of bound antigen. This binding, however, reached a maximum at a certain amount of antigen added and the addition of further antigen resulted in most cases in a decrease of the binding. The phenomenon may be explained by the dissolution of serum antibody-antigen and fixed antibody-antigen complexes by an excess of antigen.

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INTESTINAL MICROFLORA OF THE LARVAE OF ST. MARK'S FLY

II. COMPUTER ANALYSIS OF INTESTINAL ACTINOMYCETES FROM THE LARVAE OF A BIBIO-POPULATION

By

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(Received November 21, 1966)

Summary. A total of 2557 actinomycetes strains were isolated from the excrement and intestinal tract of *Bibio marci* L. larvae belonging to one population and from the control soil (rendzina A₁₄). The soil actinomycetes flora, which contained many species, was subjected to a considerable selection in the intestinal tract, where a smooth and a hairy spore surface variant of one Streptomyces species (*S. finlayi* SZABÓ *et al.* 1963 = *Act. herbiferis* KRASSILNIKOV *et* EGOROVA 1965) predominated. Other actinomycetes occurred in small numbers.

In a previous paper [1] we described the taxonomic position of "griseus" strains occurring in the intestinal tract of *Bibio* larvae. In this study we compare the species composition of the intestinal actinomycetes flora with that of the soil where the larvae are living and the organic and mineral matter of which they continuously pass through their intestinal tract.

Materials and methods

Soil. Mull-like forest rendzina formed on limestone.

***Bibio marci* L. larvae.** A total of 220 larvae of one population were dissected. The contents of the middle and hind guts were examined partly as individual, partly as pooled (mixed) samples obtained from many larvae. As qualitative and quantitative individual differences were insignificant, they will not be discussed in this paper.

Isolation of cultures was performed as described previously [1]. In addition to glucose-asparagine, glycerol-asparagine and glucose-casein agar, the synthetic basal medium of Pridham and Gottlieb supplemented with different carbon sources, was also employed.

Computer analysis. Comparison of the isolates and authentic strains was performed by means of an URAL-2 computer on the basis of 76 physiological and cultural-morphological properties. All strains were examined under rigorously identical conditions. In calculating the similarity index (S value) for each pair of strains, negative features were also regarded as similarity: $[100(a + d)] / (a + b + c + d)$, where *a* and *d* are the numbers of positive and negative characters possessed by both strains, respectively; *b* and *c* are the numbers of positive and negative features present in the first but not in the second strain and vice versa. **Cluster-analysis.** After the determination of the S-values the strains were grouped by the highest linksorting method.

Coding of features. In order to avoid an unnecessary increase of differences among the strains, quantitative variances in features were in most cases disregarded. Thus for the determination of the carbon utilization spectra only positive and negative reactions were distinguished. In some characters, however, quantitative differences had to be considered. For example, in contrast to strains never producing sporophores with more than 50 conidia, strains in the case of which the number of spores always exceeded 50, were coded with a plus character. In antibiotic resistance 3 degrees and accordingly 3 characters (A, B, C) were distinguished,

viz. resistant (—;NC;NC), moderately or less resistant (A;B;—) and highly sensitive (A;NC;C). (NC = not comparable.) Coding of the morphological structure of sporophores presented a special problem. Among various possibilities it seemed best to consider two hypothetical properties, viz. character A, which is responsible for sporulation and production of rectus flexibilis-type sporophores, and character B, which is responsible for the spiral winding of spore chains. In this respect sterile strains were not comparable (NC).

Series analysis. In the first step the strains were classified according to our section-series system [2]. In many cases several species and variants belonging to the same series were isolated. These are designated and distinguished with serial numbers within the corresponding series (Table I). Computer analysis has shown that neither the series system, nor species determination based on a few properties are always reliably reflecting the systematic position of strains.

Results

Comparison of soil, excrement and intestinal actinomycetes flora. From a mixed soil sample — the components of which were taken from several sites of an area covering approximately 1 sq.m. of the top layer of the A_H horizon situated under the investigated larval community, and from samples of excrement and intestinal contents, a total of 2557 actinomycetes strains was isolated and studied. It was also attempted to find a medium suitable for the isolation of all or at least the majority of actinomycetes species from a given sample. Table I presents the distribution of the strains, originating from the 3 different biotops, according to their series-position.

The 36 groups of *Streptomyces* strains correspond to the same number of species or variants on the basis of the so-called "important diagnostic features" employed in the classification of actinomycetes. The strains fell into 16 different series. Three further groups of strains (No. 37 to 39) were identified as *Nocardia* (*Proactinomyces*) related to species *N. alba*, *Pr. (N.) cyaneus* and *N. parafinae*. Groups No. 40 to 44 failed to show characters which would allow their classification into series. Table I shows in addition the distribution of " A_H -strains" according to the culture media on which they were isolated. These data give an account of the suitability of various media for qualitative and quantitative estimation of the actinomycetes flora. The number of strain groups (species) isolated on glycerol-arginine-agar (AGS) was 35. Other media were less effective (d-xylose 16, i-inositol 10, etc.). It is seen among the culture media, AGS agar was the most suitable for the isolation of actinomycetes. Although for the determination of the total number of germinating actinomycetes, glucose-casein-agar (see Table IV) was equivalent to AGS-agar, but the latter was more advantageous for isolation purposes since on it the colonies of the various *Streptomyces* species were growing in a characteristic and highly differentiated way. It should be noted that some strains (Griseus 2, Antibioticus 6, etc.) were isolated on other media only. These organisms were, however, so unfrequent, that their growth on some plates may have been due to chance. On the basis of these findings the examination of excrement and intestinal actinomycetes flora was carried out on AGS agar.

Table I indicates that soil (A_H) contained a much wider variety of actinomycetes series and species than the intestine and the excrement (43 vs. 6 and 10). Every actinomycetes present in the intestine was a typical soil-inhabiting microorganism. In addition to the considerable reduction in the total number of species, interesting quantitative differences were revealed. In soil many species and variants were common such as *Albosporeus* 2, *Antibioticus* 1, 2, 3, *Cinereoruber* 1, 3, *Chartreusis*, etc. In contrast, in the intestine and excrement only two *Streptomyces* (one Viridans species and members of the undetermined group No. 40) occurred in absolute predominance (over 80 per cent in both materials). Other strains isolated from excrement belonged to 8, those isolated from intestine to 4 (*Phaeochromogenes*, *Griseus*, *Flavovirens* and *Venezuelae*) different series. These organisms were found to constitute at most a few per cent of the total actinomycetes flora. Counting performed on various media indicated that actinomycetes comprised 25 to 30 per cent of the total intestinal flora and transiently 70 per cent of the total excrement flora.

Identification of intestinal and excrement actinomycetes flora with the aid of computer analysis. Classification according to series determined the approximative systematic position of our cultures. In the next step the isolates were compared partly with one another, partly with closely related authentic or preferably type strains of known species. The investigations revealed that strains belonging to the series Viridans (No. 34) and undetermined group No. 40 could be united into one group on the basis of a similarity level higher than 80 per cent. These strains were accordingly classified into one, although highly variable species. The previously undetermined isolates corresponded to forms that had lost their fertility. From this finding it follows that in reality one single *Streptomyces* species was able to exert a free multiplication in the intestine and excrement. Table II presents some examples for the determination of strains belonging to this large group.

In Table II each isolate is marked according to its origin (intestine, excrement or horizon A_H). The first two strains (4174 and 4175) and strains No. 4 to 18 (4162 to 4169) represent the population of the *Streptomyces* species which predominated in intestine and excrement. These strains were all identified as fertile members of the Viridans series (strain-group No. 34). Some closely related authentic strains are also presented. A few strains represent *Streptomyces* species which occurred in small numbers in excrement and intestine (*S. lavendulae* strains 4159 and 4172 were isolated in investigations other than those presented in Table I). Finally 3 strains isolated from horizon A_H are also shown. The holotype strain of *S. finlayi* (1869) was computed in one group with the 17 Viridans strains, in place 3. As in group "1a" *S. finlayi* 1869 and 6 Viridans strains (all included in the table as *S. finlayi*) constituted a very closely related group, they were classified into the same species. Eleven further Viridans strains fell in a group joined directly to group "1a". The members of this group ("1b")

Table I

Series distribution of actinomycetes strains isolated from the intestine and excrement of *St. Mark's fly* larvae on glycerol-arginine (AGS) agar and from control soil (A_H) on various media

Series	In- tes- tine	Ex- cre- ment	Soil (<i>A_H</i>)										
			Culture media										
			AGS	AGS	AGS	Glucose-asparagine	Glucose-casein	(NH ₄) ₂ SO ₄ -wax	(NH ₄) ₂ SO ₄ -inositol	(NH ₄) ₂ SO ₄ -raffinose	(NH ₄) ₂ SO ₄ -xylose	(NH ₄) ₂ SO ₄ -cellulose	(NH ₄) ₂ SO ₄ -sorbitol
1 Albus-Sterilis 1	—	—	10	8	17	11	35	1	37	8	15	2	1
2 Albus-Sterilis 2	—	—	12	7	3	—	—	—	—	—	—	—	—
3 Albus-Sterilis 3	—	—	2	—	1	—	—	—	—	—	—	—	—
4 Ruber-Sterilis	—	—	12	11	10	—	—	—	—	—	—	—	—
5 Violaceus-Sterilis	—	—	2	—	5	—	—	—	—	—	—	—	—
6 Phaeochromogenes 1	—	—	2	2	5	—	3	2	1	2	—	—	1
7 Phaeochromogenes 2	—	—	2	—	—	3	—	—	10	3	7	10	—
8 Phaeochromogenes 3	1	—	5	8	2	4	10	36	27	5	—	14	—
9 Albosporeus 1	—	—	8	—	4	4	—	—	—	—	—	—	—
10 Albosporeus 2	—	—	15	18	17	5	12	37	9	20	—	22	—
11 Griseus 1	2	12	2	6	3	1	—	—	1	1	—	8	—
12 Griseus 2	—	—	—	—	—	1	—	—	—	—	—	—	—
13 Antibioticus 1	—	3	19	19	10	3	—	—	30	7	10	7	12
14 Antibioticus 2	—	—	15	22	22	3	—	18	10	20	2	21	4
15 Antibioticus 3	—	2	30	35	23	—	—	3	1	—	—	—	—
16 Antibioticus 4	—	—	4	4	4	—	—	—	—	—	—	—	—
17 Antibioticus 5	—	1	2	—	—	—	—	—	—	—	—	1	—
18 Antibioticus 6	—	—	—	3	—	—	—	—	—	—	—	—	—
19 Antibioticus 7	—	—	—	2	—	—	—	—	—	—	1	—	—
20 Antibioticus 8	—	—	1	—	—	—	—	—	—	—	1	—	—
21 Cinereoruber 1	—	—	15	52	21	30	22	24	16	38	14	69	10
22 Cinereoruber 2	—	—	5	5	9	—	—	—	1	—	—	3	—
23 Cinereoruber 3	—	—	5	5	11	—	21	7	11	2	2	10	—
24 Cinereoruber 4	—	—	2	3	1	—	—	—	—	—	—	—	—
25 Violaceorectus	—	—	1	—	4	—	—	—	—	—	—	—	—
26 Flavovirens	3	10	1	1	1	—	—	—	—	—	—	—	—
27 Venezuelae 1	2	2	13	2	8	—	—	—	—	—	—	—	—
28 Venezuelae 2	—	1	—	2	1	4	—	3	5	—	—	5	—
29 Griseoflavus 1	—	—	2	6	1	—	—	—	—	—	—	—	—
30 Griseoflavus 2	—	—	—	—	1	—	—	—	—	—	—	—	—

(Table 1 continued)

Series	In- tes- tine	Ex- cre- ment	Soil (A_H)												
			Culture media												
			AGS	AGS	AGS	Glucose-asparagine	Glucose-casein	(NH ₄) ₂ SO ₄ -wax	(NH ₄) ₂ SO ₄ -inositol	(NH ₄) ₂ SO ₄ -raffinose	(NH ₄) ₂ SO ₄ -xylose	(NH ₄) ₂ SO ₄ -cellulose	(NH ₄) ₂ SO ₄ -sorbitol	(NH ₄) ₂ SO ₄ -rhamnose	Without C-source
31 <i>Griseoflavus</i> 3	—	—	2	—	2	—	—	—	—	—	—	—	—	—	
32 <i>Collinus</i> 1	—	—	1	—	1	—	—	—	—	—	—	—	—	—	
33 <i>Collinus</i> 2	—	4	—	—	—	—	—	—	—	—	—	—	—	—	
34 <i>Viridans</i>	10	174	5	1	3	—	—	—	—	—	—	—	—	—	
35 <i>Lavendulae</i>	—	—	1	1	—	1	—	—	—	—	—	—	—	—	
36 <i>Chartreusis</i>	—	—	15	40	14	29	39	49	64	36	20	54	23	—	
37 <i>Nocardia</i> (alba)	—	—	14	27	38	—	—	—	—	—	—	—	—	—	
38 <i>Nocardia</i> (paraffinae)	—	—	—	—	—	—	—	—	—	—	1	—	—	—	
39 <i>Nocardia</i> (Pr. cyaneus)	—	—	—	2	—	—	—	—	—	—	—	—	—	—	
40 Undetermined	23	49	2	1	—	—	1	—	—	—	—	—	—	—	
41 "	—	—	1	—	1	—	—	—	—	—	—	—	—	—	
42 "	—	—	23	4	31	3	3	—	15	—	7	4	—	—	
43 "	—	—	33	8	6	5	56	42	39	10	24	36	5	—	
44 "	—	—	—	2	—	—	—	—	—	1	—	—	—	—	

were less closely related to one another than the strains classified into group "1a". In view of a gradual linkage represented by strains 4163/g, 4163/x, etc. and of an inter-subgroup similarity higher than 80 per cent, group "1a" and group "1b" were considered to form one large unit of common origin, in other words the subgroups "1a" and "1b" to contain different variants of one species (*S. finlayi*). Both variants occurred in excrement and intestinal content. The relationship between the two variants is demonstrated in Table III, which shows some properties of 15 *S. finlayi* strains.

The most striking and important character which distinguishes these variants is electronmicroscopic spore surface morphology: subgroup "1a" is characterized by hairy, subgroup "1b" by smooth spore surface. Both subgroups produced "cinereus" aerial mycelium, spiral sporophore, green substrate mycelium and gave a negative Tresner—Danga reaction. All strains utilized rhamnose, maltose, dextrin, etc., as C source, but not sucrose, raffinose, inulin, inositol, sorbitol, etc. With xylose, lactose, etc., different reactions were observed in both subgroups. Similarly, no subgroup-characteristic properties were revealed by other reactions (growth in the presence of 6 per cent KI, etc.).

Table III
Comparison of some *S. finlayi* strains on the basis of 33 characters

Designation of strains	Characters
1. <i>S. finlayi</i> 4174	Spi; Sm ^{gr} ; T-D ⁻ ; Ha; L ^c ; Nit ⁻ ; col ⁻ ; sub ⁻ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K ⁺ ; 25Mg ⁺ ; Ga ⁺ ;
2. <i>S. finlayi</i> 4175	Spi; Sm ^{gr} ; T-D ⁻ ; Ha; L ^c ; Nit ⁻ ; col ⁻ ; sub ⁻ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K ⁺ ; 25Mg ⁺ ; Ga ⁺ ;
3. <i>S. finlayi</i> 4161	Spi; Sm ^{gr} ; T-D ⁻ ; Ha; L ^c ; Nit ⁻ ; col ⁻ ; sub ⁻ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁺ ; 6K ⁺ ; 25Mg ⁺ ; Ga ⁺ ;
4. <i>S. finlayi</i> 4162	Spi; Sm ^{gr} ; T-D ⁻ ; Ha; L ^c ; Nit ⁻ ; col ⁻ ; sub ⁻ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K ⁺ ; 25Mg ⁺ ; Ga ⁺ ;
5. <i>S. finlayi</i> 4163	Spi; Sm ^{gr} ; T-D ⁻ ; Sh; L ^c ; Nit ⁺ ; col ⁻ ; sub ⁻ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K ⁺ ; 25Mg ⁺ ; Ga ⁺ ;
6. <i>S. finlayi</i> 4164	Spi; Sm ^{gr} ; T-D ⁻ ; Sh; L ^c ; Nit ⁺ ; col ⁻ ; sub ⁻ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K [?] ; 25Mg ⁺ ; Ga ⁺ ;
7. <i>S. finlayi</i> 4200	Spi; Sm ^{gr} ; T-D ⁻ ; Sh; L ^c ; Nit ⁺ ; col ⁻ ; sub ⁺ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K ⁻ ; 25Mg ⁺ ; Ga ⁺ ;
8. <i>S. finlayi</i> 4165	Spi; Sm ^{gr} ; T-D ⁻ ; Sh; L ^c ; Nit ⁺ ; col ⁻ ; sub ⁺ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K ⁺ ; 25Mg ⁻ ; Ga ⁺ ;
9. <i>S. finlayi</i> 4166	Spi; Sm ^{gr} ; T-D ⁻ ; Sh; L ^c ; Nit ⁺ ; col ⁻ ; sub ⁺ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K [?] ; 25Mg ⁻ ; Ga ⁺ ;
10. <i>S. finlayi</i> 4167	Spi; Sm ^{gr} ; T-D ⁻ ; Sh; L ^c ; Nit ⁺ ; col ⁻ ; sub ⁺ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K ⁺ ; 25Mg ⁺ ; Ga ⁺ ;
11. <i>S. finlayi</i> 4169	Spi; Sm ^{gr} ; T-D ⁻ ; Sh; L ^c ; Nit ⁺ ; col ⁻ ; sub ⁺ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁺ ; 6K ⁺ ; 25Mg ⁺ ; Ga ⁺ ;
12. <i>S. finlayi</i> 4171	Spi; Sm ^{gr} ; T-D ⁻ ; Sh; L ^c ; Nit ⁺ ; col ⁺ ; sub ⁻ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K ⁺ ; 25Mg ⁺ ; Ga ⁺ ;
13. <i>S. finlayi</i> 4201	Spi; Sm ^{gr} ; T-D ⁻ ; Sh; L ^c ; Nit ⁺ ; col ⁻ ; sub ⁺ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K ⁻ ; 25Mg ⁺ ; Ga ⁺ ;
14. <i>S. finlayi</i> 4163/g	Spi; Sm ^{gr} ; T-D ⁻ ; Sh; L ^c ; Nit ⁻ ; col [?] ; sub [?] ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K ⁺ ; 25Mg ⁺ ; Ga ⁺ ;
15. <i>S. finlayi</i> 1869	Spi; Sm ^{gr} ; T-D ⁻ ; Ha; L ^c ; Nit ⁻ ; col ⁻ ; sub ⁻ ; 40 ^o ; 5 ^o +; Hae ⁻ ; pH4 ⁻ ; 6KJ ⁻ ; 6K ⁺ ; 25Mg ⁺ ; Ga ⁺ ;

Abbreviations: Spi = spiral; Sm^{gr} = substrate mycelium green in colour; T-D⁻ = negative Tresner-Danga reaction; spore surface (electronmicroscopic): Ha = hairy, Sh = smooth; L^c = aerial mycelium: cinereus; Nit = nitrate reduction; col = antibiotic activity against *E. coli*; sub = inhibition of *B. subtilis*; 40^o or 5^o = growth at the corresponding temperature; Hae = haemolysis; pH4 = growth at pH 4; 6 KI, 6 K, 25 Mg = growth in the presence of 6% KI, 6% KCl, 25% MgSO₄ · 7H₂O; carbon source utilization + or -: Ga = galactose; Fr = d-fructose; Xy = d-xylose; Rh = rhamnose; Ar = l-arabinose; Sa = sucrose; Mal = maltose; La = lactose; Raf = raffinose; Inu = inulin; In = i-inositol; Sor = sorbitol; Ma = d-mannitol; De = dextrin; Sal = salicin; Ze = cellulose; W = wax; Par = paraffin.

Table III continued

Designation of strains	Characters															
1. <i>S. finlayi</i> 4174	Fr ⁻	Xy ⁺	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁺ ; Par ⁻
2. <i>S. finlayi</i> 4175	Fr ⁻	Xy [?]	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁻ ; Par ⁻
3. <i>S. finlayi</i> 4161	Fr ⁻	Xy ⁺	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁺ ; Par ⁻
4. <i>S. finlayi</i> 4162	Fr ⁻	Xy ⁺	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁺ ; Par ⁻
5. <i>S. finlayi</i> 4163	Fr ⁻	Xy ⁺	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁺	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁺ ; Par ⁻
6. <i>S. finlayi</i> 4164	Fr ⁻	Xy ⁻	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁻ ; Par ⁻
7. <i>S. finlayi</i> 4200	Fr ⁻	Xy [?]	Rh ⁺	Ar [?]	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁺ ; Par ⁻
8. <i>S. finlayi</i> 4165	Fr ⁻	Xy ⁻	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁺ ; Par ⁻
9. <i>S. finlayi</i> 4166	Fr ⁻	Xy ⁺	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁺ ; Par ⁻
10. <i>S. finlayi</i> 4167	Fr ⁺	Xy ⁺	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁺ ; Par ⁻
11. <i>S. finlayi</i> 4169	Fr ⁺	Xy ⁺	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁺	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁺ ; Par ⁻
12. <i>S. finlayi</i> 4171	Fr ⁻	Xy ⁺	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁺ ; Par ⁻
13. <i>S. finlayi</i> 4201	Fr ⁻	Xy ⁻	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W ⁺ ; Par ⁻
14. <i>S. finlayi</i> 4163/g	Fr ⁻	Xy ⁺	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W [?] ; Par ⁻
15. <i>S. finlayi</i> 1869	Fr ⁻	Xy ⁺	Rh ⁺	Ar ⁺	Sa ⁻	Mal ⁺	La ⁻	Raf ⁻	Inu ⁻	In ⁻	Sor ⁻	Ma ⁻	De ⁺	Sal ⁻	Ze ⁻	W [?] ; Par ⁻

Table II shows that authentic strains *S. viridogenes* ATCC 3372, *S. viridis* INA 3372, *S. flavovirens* IPV 3 and ATCC 3320, which had been derived partly from one parent strain, were computed beside the two *S. finlayi* subgroups. Group "2" formed by these cultures is followed by group "3" containing "griseus" strains. The next group ("4") is constituted of two typical green *Streptomyces* isolated from humus horizon, *S. olivaceus* ATCC 3335 and some "type" cultures of green *Actinomyces* (*Streptomyces*) species described by GAUZE *et al.* Group "5", "6" and "7" contained strains showing a gradually decreasing relationship with green streptomycetes. The relatively scattered positions occupied by *S. olivaceus* strains is explained by the fact that the original identification of these strains had been performed on the basis of so-called "diagnostically important characters" [3]. *Streptomyces* which occurred in small numbers in excrement and intestine belonged to different species. Table II refers to their systematic position merely in view of their relation to the predominant *S. finlayi* strains.

Estimation of the number (population) of individual actinomycetes species in the total microbial community by use of a selective method. Table IV, derived partly from data of Table I, shows the richness in actinomycetes species and variants of the 3 investigated substrata as well as the total amount of these organisms in the excrement and the A_H -horizon. A comparison of relative AGS agar counts for A_H (100) and for excrement (251) indicates that the actinomycetes content of excrement was about 2 1/2 times that of soil (22.1 vs. 8.8 million/g dry weight). The unusually high actinomycetes content of excrement was attributed to the intensive multiplication of *S. finlayi*. The incidence of this organism in excrement exceeded transiently 90 per cent of the total actinomycetes flora.

If one or a few species are present in the microflora, their number can be estimated by counting morphologically identical colonies on a suitable medium. In the presence of many relatively frequent species (A_H horizon), this method is hardly applicable. When a suspension of A_H soil sample is seeded on agar plates containing various C and N sources, germination of certain species is inhibited selectively. By considering the number of colonies developing and not developing on these media, we were able to estimate the population number of distinct smaller groups consisting of a few species and sometimes even of one single species. For example, *N. alba* belonging to the series "Alba" of *Nocardia*, produced a large number of characteristic white, soft colonies in the presence of organic N sources, but failed to develop on a medium containing ammonium sulphate as a sole source of nitrogen. Direct counting and indirect estimation based on the lack of growth gave approximately similar results, i.e. 6.5 million cells per 1 g dry soil. In the upper 1 to 5 cm layer of horizon A_H situated under the activity area of the larval population, *N. alba* was the absolute predominant organism. All strains of

The table of the S values (%) for the compared streptomycetes (authentic strains and our isolates) after re-arranging the strains by cluster analysis

[illegible]

S. chartreusis of the *Chartreusis* series were able to produce, even in the absence of a carbon source, little point-like colonies with woolly secondary aerial mycelium. Total counts for this organism were easy to estimate on PRIDHAM—GOTTLIEB's C control basal medium, as among the 6 accompanying species (see Table I) 4 were accidental and 2 (No. 13 and 21) were uncommon organisms with entirely different morphological properties. *S. chartreusis* occurred in the range of 20 to 25 thousand/g soil. On wax, 15 species and variants were detected (Table IV). These organisms were partly unfrequent wax-utilizing

Table IV

Qualitative and quantitative distribution of actinomycetes in excrement and gut content of Bibio larvae and in control soil

Culture medium	Source of strains	Total amount of strains	Number of species or variants		Actinomycetes, thousands per gm dry soil	
			No	%	$\bar{X}$	Rel
AGS	Excr.	258	10	28	22,150	251
AGS	gut content	41	6	17	NCD	NCD
AGS	Soil	284	35	100	8,815	100
Glucose-asparagine	"	307	30	86	7,750	88
Glucose-casein	"	280	32	91	8,900	101
Wax	"	107	15	43	96	1.1
Inositol	"	202	10	28	200	2.3
Raffinose	"	222	11	31	413	4.7
Xylose	"	277	16	46	85	0.9
Cellulose	"	153	13	37	107	1.2
Sorbitol	"	104	12	34	95	1.1
Rhamnose	"	266	15	43	1,965	22.3
Without C source	"	56	7	20	27	0.3

NCD = non-comparable data

variants of 11 different species. As to the remaining 4 actinomycetes, *S. chartreusis* counts are presented above; organisms belonging to series *Albus-Sterilis* 1, *Antibioticus* 1 and *Cinereoruber* 1 were found in total counts of 65 to 70 thousand/g soil. Since the utilization of carbon sources was not uniform even with strains of the same population of a species, for total count determination a carbon source supporting the growth of the largest number of variants within a given species should be used.

General ecological-physiological description of the intestinal actinomycetes flora. Comparative numerical-taxonomic analysis of microflora and populations of individual species also supplied detailed information on the general

ecophysiological character of the organisms examined. *S. finlayi*, although a thermotolerant organism, grows readily at low temperatures (above 6°C) and is unable to develop at 38°C. Its vegetative mycelium is fairly sensitive to dry and moist heat. It is interesting that during the winter *Bibio* larvae feed even on frozen soil. *S. finlayi* strains utilized cellulose weakly or not at all. Most of them grew at pH 9.0. Although, as shown by examinations on LIESKE's protein agar, they considerably differed in proteolytic activity, they probably play an important role in the decomposition of proteins in the gut. *S. finlayi* strains are able to utilize most amino acids as sole N-sources in the medium. The less frequently utilized amino acids are l-cystine, dl-methionine and l-tyrosine. In synthetic media these strains produce many kinds of free amino acids: α -alanine, lysine, threonine, glycine, aspartic acid, arginine; some strains also valine and leucine. Certain strains decompose waxy materials, but none of them can utilize paraffins. The holotype strain does not grow on wax. Not all the strains reduced nitrates to nitrites. Salt tolerance of our organisms was of a medium degree. Several strains grew in the presence of 25 per cent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 per cent NaCl, 6 per cent KCl or 6 per cent KI. None of the strains tolerated 25 per cent $\text{Na}_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$. Production of antibiotics effective against Gram negative bacteria was rarely observed. A weak antagonism against Gram positive test cultures, however, was frequently demonstrated. All strains were resistant to penicillin, polymyxin-B and erythromycin. The cultures considerably differed in sulphonamide, chloramphenicol, aureomycin, terramycin and tetracycline sensitivity (resistant to highly sensitive). None of the strains fixed atmospheric nitrogen. As regards a possible humification in the intestine, *S. finlayi* strains or their disintegrated mycelium or plasma fragments play probably no active part (negative TRESNER—DANGA reaction, failure to produce melanoid substances, negative polyphenol oxidase reaction).

Discussion

Actinomycetes seem to be important members of the intestinal flora not only in earthworms but in soil-inhabiting insect larvae ingesting organic materials of the soil. The soil actinomycetes flora, which consists of several species and variants, is subjected to considerable selection in the intestine. Effective intestinal multiplication and, consequently, an absolute predominance (over 80 per cent) in the intestinal actinomycetes flora was noted for one species. This species showed a transient further increase in the excrement of the examined larvae. Later, however, it was replaced by the multiple species population characteristic of soil microflora.

Computer analysis, now extensively applied in bacteriological taxonomy [5], seems advantageous in the determination of actinomycetes [6] and especially in the analysis of populations of individual species. As it has

been demonstrated, the dominant members of the intestinal flora (organisms undeterminable by series analysis and Viridans strains) were computed in one large group which may be considered a variable population of one of the same species (*S. finlayi*). In this *S. finlayi*-population constituted partly by fertile strains partly by sterile ones we separated two subgroups on the basis of a great number of features. The fertile strains of these two subgroups (*S. finlayi* variants "1a" and "1b"; see Table II) were distinguished by their different spore surface morphology. The fact that one population of a species contained variants of hairy and smooth spore surfaces indicates that this morphological character, which has been regarded very stable, is in reality variable. Our observation confirms the finding of DOLEŽILOVÁ *et al.* [7] who described the production of spiny and smooth spore types in one and the same *S. erythraeus* strain.

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INTESTINAL MICROFLORA OF THE LARVAE OF ST. MARK'S FLY

III. COMPUTER ANALYSIS OF THE INTESTINAL ACTINOMYCETES-FLORA OF VARIOUS LARVAL POPULATIONS

By

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(Received November 21, 1966)

Summary. The intestinal microflora of 508 larvae from 6 different *Bibio marci* L. populations has been compared. *Streptomyces* constituted 15—20 to 50 per cent of the total intestinal microflora. There was no significant individual difference in the qualitative composition of intestinal *Streptomyces*-flora in larvae derived from one population. The absolute predominance of one species and the presence of several other species in small numbers was characteristic. Computer analysis based on 84 features indicated that predominating (otherwise typical soil inhabiting) *Streptomyces* species varied with different larval populations. Analysis of 319 intestinal *Streptomyces* strains showed that the representatives of the highly variable species *S. finlayi* SZABÓ et al. 1963 (syn.: *Act. herbiferis* KRASSILNIKOV et EGOROVA 1965) predominated in the gut of the larvae of 3 different *Bibio* populations, and each of the further 3 larval communities had its own predominating *Streptomyces* belonging to the species *S. olivaceus*, *antibioticus* and *aureofaciens*.

In the intestinal actinomycetes-flora of *Bibio* larvae derived from one "Fressgemeinschaft" (larval population) the predominant occurrence of *S. finlayi* and small number of other *Streptomyces* species differed considerably from the actinomycetes flora of the control soil, which was characterized by a great number of the different and partly very common species. This finding called the attention to the "specificity problem" of the intestinal microflora in lower animals feeding on decomposing organic materials of the soil and of other substrates. Although since JENSEN's studies in 1898 the problem was subjected to investigation by several authors (Table I), it has remained unsolved.

The studies have shown that the total number of microorganisms increases in the gut and the excrement of the animals [3—7] and an alteration occurs in the composition of the microflora; while certain organisms disappear, others increase in number [8—13]. The enzymatic activity of excrement increases parallel with the increase of microbial activity [14]. The animals may instinctively select the material serving as their food according to its bacterial content [15, 16]. The facultative pathogenicity of certain soil microbes has also been described [17, 18]. The intestinal microflora contains common saprophytic soil microbes in high numbers [19]. A close symbiosis has been revealed between the host and certain cellulose-decomposing bacteria [20, 21].

Table I

A summary of data for the microflora in intestine and excrement of worms and arthropods inhabiting soil and decomposing materials

Author	Year	Materials	Organisms	Short summary of the results
Jensen	1898	Soil + <i>B. stutzeri</i>	Earthworms	Denitrifying bacteria were destroyed in the intestinal canal; their occurrence in the excrements was rare.
Basalik	1913	Soil	Earthworms	The total number of microbes was higher in the excrements than in the soil, but the qualitative composition of the microflora of these two substrates was not different.
Aichberger	1914	—	Earthworms	No viable yeasts, rhizopodes, diatoms, blue-green algae and desmids were found in the alimentary canal.
Vaternahm	1924	Horse-dung	Geotrupes sp.	No microflora specific for the alimentary canal was demonstrated
Werner	1926	Ant-hill	<i>Potosia cuprea</i> larvae	Cellulose-digesting bacteria (<i>B. cellulosa fermentans</i>) are present in special chambers of the intestine.
Buchner	1930	—	<i>Tipula maxima</i> larvae	A blind sac connected to the colon contains large numbers of <i>B. cellulosa fermentans</i> .
Madle	1934	Cow-dung	<i>Aphodius rufipes</i>	Staphylococcal, coli and yeast strains were isolated from the intestinal content of larvae as well as from cow-dung. These played no role in the animal's digestive function
Day	1950	Loam	Earthworms	The total number of microbes showed no consistent difference in fresh castings of <i>L. terrestris</i> and soil. <i>S. marcescens</i> was completely destroyed in the earthworm.
Ruschmann	1953	Cultivated soil, compost soil	Earthworms	The total number of microorganisms in excrements of <i>Eisenia</i> and <i>Lumbricus</i> species was higher than in control soil. Actinomycetes were predominating.
Ponomareva	1953	Podzol soil	Earthworms	The number of actinomycetes and <i>B. cereus</i> group bacteria was increasing during passage through the intestinal canal.
Schütz, Felber	1956	Ground cowdung	Earthworms	Actinomycetes : bacteria : fungi relation was 10 : 6 : 1.4 in the food and 10 : 4 : 0.7 in the excrement of <i>Eisenia foetida</i> .
Kiss	1957	Brown forest soil	Formicoidea Earthworms	Enzymatic (saccharase) activity in <i>Lumbricus terrestris</i> excrement was higher than in the control soil.

Khambata, Bhat	1957	—	Earthworms	The normal intestinal flora of a <i>Pheretima</i> sp. consisted of pseudomonads coryneform bacteria, nocardias, streptomycetes and spore-forming bacilli.
Stebayev	1958	Tundra soil	Tipula larvae	<i>B. mesentericus</i> was common in the excrement; fungi occurred in small numbers. Pseudomonad counts were 12 times higher in the excrement than in the soil. The two materials showed no difference as to the number of oligonitrophilic bacteria.
Brüsewitz	1959	Model soils	Earthworms	The richness or poverty of soil in nutrients showed a reversed relation to the total number of faecal microbes. A selective influence of intestinal passage was demonstrated.
Kurcheva	1960	Oak leaf fall, chernozem	<i>Tipula scripta</i> , Oniscoidea	Humus formation occurs as a result of mutual activity of the animals and microorganisms.
Führer	1961	Sandy soil	Oribatei	A <i>Pseudomonas</i> strain from <i>Artemisia</i> roots possessed a strong attractive power on <i>Pseudotritia ardua</i> common in the <i>Artemisia</i> rhizosphaera.
Kozlovskaya, Zhdannikova	1961	Taiga forest podzol	Earthworms	Actinomycetes and spore-forming bacteria (<i>B. cereus</i> , <i>B. idosus</i>) are predominant in faeces.
Kring	1962	Soil	Elaterida larvae	The larvae were destroyed by toxic metabolic products of <i>Ps. fluorescens</i> growing on green manure.
Karg	1963	Light and heavy soils	Acarina	Certain species are specialized to feed on soil microbes.
Went	1963	Clay soil	Earthworms	The increase in the number of bacteria in the excrements is caused by the soil mixing with organic matter of the top layer.
Törne	1963	Raw humus, wheat straw	Collembola	Specific composition of the microflora in the substrate highly influences the substrate-choosing activity of the animals.
Szabó, Marton, Pártai	1964	Mull-like rendzina	Diptera larvae, Earthworms	Direct microscopic observations indicate that the excrements of various animals contain different microbial populations.
Stebaev, Naplekova, Gukasian	1964	Dry steppes on light chestnut soils	Tenebrionidae, Acrididae	The faecal microflora varies with the quality of food and the metabolic activity of the animal. The isolated cellulose bacteria were <i>Sporocytophaga</i> , <i>Sorangium</i> and <i>Vibrio</i> species.

The microbial content of faeces may vary with the food of the animal [22]. Food and faeces may contain an identical microflora [23, 24]. Some authors suppose that microorganisms are responsible for humification in the intestine [25].

The present paper deals with the problem of organisation and selection of microbial communities in the intestinal milieu, based upon studies in the larvae of St. MARK's fly.

Materials and methods

Larval populations. Colonies containing 500–2000 individuals were collected from mull-like rendzina soil. All larvae belonged to the species *Bibio marci* L. Both larvae and bred images were determined, the former on the basis of the microscopical cuticular structure of the abdominal and thoracic segments.

Isolation of actinomycetes was performed on glycerol-arginine (AGS) agar [1].

Computer analysis. Percentage of similarity was estimated on the basis of 84 features. The principle of "cluster analysis" has been described previously [1]. Calculations were performed by means of the URAL-2 electronic computer.

Results

The intestinal content of a large number of larvae was examined on peptone-, glucose-casein- and glucose-asparagine agar by plating. In addition to the 6 populations described in this paper, several other larval colonies were examined. It was shown that actinomycetes constitute somewhat more than 20 per cent of the total intestinal microflora. Sometimes their incidence reached 50 per cent, or was below 20 per cent. Among larval populations No. 1 to 6 in Table II, No. 6 was identical with the previously described population [1]. Examination of pooled intestinal samples of each population showed that actinomycetes occurred in the intestinal content of populations 1 to 5 at 46, 18, 20, 15, and 22 per cent frequency, respectively. The 15 per cent incidence was the lowest value ever observed in our investigations.

The species composition of intestinal actinomycetes flora was investigated partly by counting colonies with identical characters appearing after plating on AGS agar, partly by comparing and systematical determination of pure isolates. Table II presents the series distribution of 319 strains, which had been isolated from pooled intestinal samples of the individual larval populations and were subjected to a thorough examination. The groups of strains (species), the representatives of which were absolute predominant members of the intestinal actinomycetes flora, are marked with asterisks. In the case of larval population No 6. the absolute predominance of *S. finlayi* has already been described; more than 80 per cent of actinomycetes colonies grown on AGS agar belonged to this species. *S. finlayi* (series Viridans) occurred at a similar frequency in the gut of larvae belonging to populations 3 and 4. On the other

hand, this species was absent in the larvae from population 1, and occurred sporadically in populations 2 and 5. The intestinal *Streptomyces* flora of these *Bibio* larval communities was characterized by the absolute predominance of other species. In systematic position these organisms differed sharply from *S. finlayi* and from one another. They belonged to series *Antibioticus*-6 (population 1), *Griseoflavus*-1 (population 5) and *Antibioticus*-? (population 2). Serial numbers in Table II (bracketed figures) denote the same species (group of identical strains) as in Table I of the previous paper [1]. Due to the fact that all 6 larval populations were collected in the same area from a mull-like rendzina soil characterized by a similarly constituted actinomycetes flora, in the present study only two groups of strains (species) were isolated which could not be included in any of the previously encountered species. These groups are denoted in Table II with question marks instead of serial numbers. One of them (*Antibioticus*-?) constituted the predominant member of the intestinal actinomycetes flora in *Bibio* population No. 2. From the gut flora of every larval community we have isolated — beside the predominant forms — several additional *Streptomyces* species, but in small numbers amounting to at most a few per cent of the intestinal actinomycetes population. For example, in the gut flora of the *Bibio*-population No. 3 at moderate frequency there occurred, in addition to the predominant *S. finlayi*, three *Streptomyces* species belonging to series *Albosporeus*-2, *Griseus* and *Antibioticus*-. *Antibioticus*-? occurred also in the intestinal microflora of population 1. The incidence of 6 further *Streptomyces* species (*Antibioticus*-3, *Cinereoruber*-1, *Venezuelae*-1, *Griseoflavus*-3 and two undetermined) was low.

In subsequent examinations the predominant *Streptomyces* species were identified. Table III shows the comparison of some strains of these species, on the basis of a number so-called "diagnostically important" characters. The larvae of 5 *Bibio* populations (I—V) yielded 4 predominating species, *S. olivaceus*, *antibioticus*, *aureofaciens*, and *finlayi*. Strains belonging to the first three species exhibited a less variable character than *S. finlayi* which was analysed previously [1]. Although *S. antibioticus* strains varied in their capacity to growth in 6 per cent KI, and *S. olivaceus* strains in NaNO_3 utilization, etc., they were uniform in most properties. For example, *S. olivaceus* strains varied only in one out of the 34 characters presented in Table III. The same was the case with *S. aureofaciens*. *S. finlayi* strains isolated from the larvae of *Bibio*-populations 3 and 4, similarly to *S. finlayi* cultures originating from larval community No. 6 [1] showed considerable variations. Smooth and hairy spore surfaces as well as variations in physiological properties (utilization of d-fructose, sucrose, raffinose and paraffin, antibiotic sensitivity, etc.) were frequent.

All strains were similar in spore colour. The full-grown, well sporulating aerial mycelium showed various hues characteristic of the "cinereus" (gray)

Table II

Distribution of Streptomyces strains belonging to various series isolated from the gut of larvae of 6 different Bibio populations

Larval population	No. of dissected larvae	Albus-Sterilis 2 (2)	Phaeochromogenes 2 (7)	Phaeochromogenes 3 (8)	Albosporeus 2 (10)	Griseus 1 (11)	Antibioticus 1 (13)	Antibioticus 2 (14)	Antibioticus 3 (15)	Antibioticus 6 (18)	Cinereoruber 1 (21)	Cinereoruber 3 (23)	Flavovirens (26)	Venezuelae 1 (27)	Griseoflavus 1 (29)	Griseoflavus 2 (30)	Griseoflavus 3 (31)	Viridans (34)	(Nocardia alba) (37)	Undetermined (42)	Undetermined (43)	Antibioticus (?)	Undetermined (?)
1	137	1	—	—	3	—	4	1	—	72*	—	3	—	—	—	—	—	—	—	—	—	13	—
2	40	—	2	—	8	—	4	—	—	1	—	—	—	—	1	3	—	2	—	—	2	14*	—
3	50	—	—	—	12	9	—	—	1	—	3	—	—	1	—	—	2	33*	—	4	—	9	2
4	41	—	—	—	—	5	—	—	1	3	1	—	—	3	—	—	—	20*	—	1	—	—	—
5	20	1	—	—	—	—	—	—	—	—	—	—	—	—	25*	—	—	7	1	—	—	—	—
6	220	—	—	1	—	2	—	—	—	—	—	—	3	2	—	—	—	33*	—	—	—	—	—

Table III

Comparison of some *Streptomyces* strains common in the intestine of *Bibio* larvae, on the basis of 34 features

Larval population	<i>Streptomyces</i>	Designation of strain	Sporophore	No. of spores	Colour of substrate mycelium	Colour of aerial mycelium (TRESNER-BACKUS)	Spore surface (electr. opt.)	TRESNER-DANGA reaction	Nitrate reduction	Inhibition of <i>E. coli</i>	Inhibition of <i>B. subtilis</i>	Growth at 40°C	Growth in 6% KI	Growth in 6% KCl	Growth in 12% Na ₂ S ₂ O ₃	Ur utilization	NaNO ₃ utilization	d-Fructose	d-Xylose	Rhamnose	l-Arabinose	Sucrose	Raffinose	i-Inositol	d-Mannitol	Dextrin	Salicin	Cellulose	Wax	Paraffin	Penicillin	Streptomycin	Chloramphenicol	Erythromycin	Terramycin	Tetracyclin
I	<i>olivaceus</i>	B-57	Rect	10+	Y	e 265	Sm	neg	+	-	-	-	-	+	-	+	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	++	++	-	++	++
I	<i>olivaceus</i>	B-59	Rect	10+	Y	e 265	Sm	neg	+	-	-	-	?	+	-	+	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	++	++	-	++	++
I	<i>olivaceus</i>	B-87	Rect	10+	Y	e 265	Sm	neg	+	-	-	-	-	+	-	+	+	-	-	+	-	-	-	-	+	-	-	+	-	-	-	++	++	-	++	++
I	<i>olivaceus</i>	B-92	Rect	10+	Y	e 265	Sm	neg	+	-	-	-	-	+	-	+	?	-	-	+	-	-	-	-	+	-	-	+	-	-	-	++	++	-	++	++
I	<i>olivaceus</i>	B-99	Rect	10+	Y	e 265	Sm	neg	+	-	-	-	-	+	-	+	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	++	++	-	++	++
II	<i>antibioticus</i>	53917	Rect	50+	Y	3 fe	Sm	poz	-	-	-	-	?	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	++	++	+	+
II	<i>antibioticus</i>	53928	Rect	50+	Y	3 fe	Sm	poz	-	-	-	-	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	++	++	+	+
II	<i>antibioticus</i>	53959	Rect	50+	Y	3 fe	Sm	poz	-	-	-	-	-	+	+	-	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	++	++	+	+
II	<i>antibioticus</i>	53966	Rect	50+	Y	3 fe	Sm	poz	-	-	-	-	-	+	+	-	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	++	++	+	+
II	<i>antibioticus</i>	53977	Rect	50+	Y	3 fe	Sm	poz	-	-	-	-	-	+	+	-	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	++	++	+	+
V	<i>aureofaciens</i>	6x	Spi	50+	Y	3 fe	Sm	neg	+	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	+	++	-	-
V	<i>aureofaciens</i>	7x	Spi	50+	Y	3 fe	Sm	neg	+	-	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	+	++	-	-
V	<i>aureofaciens</i>	8x	Spi	50+	Y	3 fe	Sm	neg	+	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	+	++	-	-
V	<i>aureofaciens</i>	9x	Spi	50+	Y	3 fe	Sm	neg	+	-	-	-	?	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	+	++	-	-
V	<i>aureofaciens</i>	10x	Spi	50+	Y	3 fe	Sm	neg	+	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	+	-	+	+	++	-	-
IV	<i>finlayi</i>	10x-IV	Spi	10+	Y-G	5 ih	Ha	neg	+	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	+	-	-	+	-	-	+	+
IV	<i>finlayi</i>	11x	Spi	10+	Y-G	5 ih	Ha	neg	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	+	-	-	+	+
IV	<i>finlayi</i>	12x	Spi	10+	Y-G	5 ih	Ha	neg	+	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	+	-	-	+	-	-	+	+
IV	<i>finlayi</i>	13x	Spi	50+	Y-G	5 ih	Sm	neg	+	-	-	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-	-	+	-	-	+	-	-	+	+
IV	<i>finlayi</i>	14x	Spi	50+	Y-G	5 ih	Sm	neg	+	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	+	-	-	+	+	-	+	+
III	<i>finlayi</i>	1x	Spi	50+	Y-G	5 ih	Sm	neg	+	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	+	-	-	+	++	-	+	+
III	<i>finlayi</i>	2x	Spi	10+	Y-G	5 ih	Ha	neg	+	-	-	-	+	+	+	+	+	+	+	+	+	?	-	-	-	+	-	-	+	-	-	+	-	-	+	+
III	<i>finlayi</i>	3x	Spi	50+	Y-G	5 ih	Sm	neg	+	-	-	-	+	+	+	+	+	+	-	+	+	+	-	-	-	+	-	-	+	-	-	+	-	-	+	+
III	<i>finlayi</i>	4x	Spi	50+	Y-G	5 ih	Sm	neg	+	-	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-	+	+
III	<i>finlayi</i>	5x	Spi	50+	Y-G	5 ih	Sm	neg	+	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	+	-	-	+	-	-	+	+

Table IV

Percentage similarity of some representative strains of *Streptomyces* species predominating in the intestinal flora of 6 different larval populations, as computed on the basis of 84 features

<i>S. finlayi</i>	10x-IV	16	100											1a																																								
<i>S. finlayi</i>	12x	18	99	100																																																		
<i>S. finlayi</i>	11x	17	98	98	100																																																	
<i>S. finlayi</i>	2x	12	98	98	98	100																																																
<i>S. finlayi</i>	4161	27	95	95	94	95	100																																															
<i>S. finlayi</i>	4174	31	94	94	93	92	98	100																																														
<i>S. finlayi</i>	4162	26	94	94	93	94	98	99	100																																													
<i>S. finlayi</i>	1x	11	92	92	90	93	91	88	90	100											1b																																	
<i>S. finlayi</i>	14x	20	92	92	90	90	89	87	87	96	100																																											
<i>S. finlayi</i>	5x	15	90	90	89	91	87	84	85	95	93	100																																										
<i>S. finlayi</i>	4169	30	93	93	91	91	88	88	87	93	93	94	100																																									
<i>S. finlayi</i>	4167	29	91	91	90	90	87	90	88	91	91	92	98	100																																								
<i>S. finlayi</i>	4200	32	85	85	84	86	88	89	89	91	88	89	92	94	100																																							
<i>S. finlayi</i>	4165	28	84	84	83	86	87	89	89	90	89	88	91	93	98	100																																						
<i>S. finlayi</i>	4x	14	85	85	84	86	84	81	83	91	88	92	86	85	84	83	100																																					
<i>S. finlayi</i>	3x	13	85	85	84	86	84	81	83	91	88	92	86	85	84	83	97	100																																				
<i>S. finlayi</i>	13x	19	87	87	85	85	81	80	80	89	89	91	88	86	79	79	95	95	100																																			
<i>S. aureofaciens</i>	10x	25	65	65	64	63	64	63	63	67	70	67	69	67	61	62	73	73	77	100											2																							
<i>S. aureofaciens</i>	7x	22	64	64	62	65	64	71	63	68	69	71	70	69	66	66	74	72	73	95	100																																	
<i>S. aureofaciens</i>	9x	24	66	66	65	64	65	65	65	68	71	68	70	70	64	64	74	71	76	98	97	100																																
<i>S. aureofaciens</i>	8x	23	65	65	64	66	66	63	64	69	70	69	69	67	65	65	76	73	74	97	98	98	100																															
<i>S. aureofaciens</i>	6x	21	65	65	64	66	66	63	64	69	70	69	69	67	65	65	76	73	74	97	98	98	99	100																														
<i>S. antibioticus</i>	53917	6	68	68	67	69	74	73	74	76	74	71	73	73	72	72	71	68	69	78	80	80	81	81	100											3																		
<i>S. antibioticus</i>	53966	9	65	65	64	66	71	72	72	73	70	68	72	73	73	72	67	64	65	74	76	77	77	77	97	100																												
<i>S. antibioticus</i>	53959	8	69	69	67	70	75	73	73	76	74	72	76	75	74	74	71	68	69	76	77	77	79	79	97	96	100																											
<i>S. antibioticus</i>	53928	7	66	66	65	67	72	73	73	74	71	69	73	75	74	74	68	65	67	76	77	78	79	79	98	98	97	100																										
<i>S. antibioticus</i>	53977	10	67	67	66	68	73	72	72	75	73	71	75	73	73	72	69	67	68	77	79	78	80	80	98	97	98	98	100																									
<i>S. olivaceus</i>	B-87	3	63	63	61	63	67	69	69	68	68	65	66	67	72	72	67	67	63	59	58	59	59	59	63	63	65	65	63	100											4													
<i>S. olivaceus</i>	B-59	2	63	63	62	61	67	68	67	66	69	63	67	67	69	69	65	65	64	57	56	59	57	57	60	60	63	62	62	97	100																							
<i>S. olivaceus</i>	B-92	4	61	61	59	61	65	67	67	66	66	63	64	65	70	70	65	65	61	55	56	57	57	57	61	62	63	63	62	98	97	100																						
<i>S. olivaceus</i>	B-57	1	60	60	58	61	64	66	66	65	65	62	63	64	71	72	64	64	60	54	55	56	57	57	60	61	62	62	61	97	97	97	100																					
<i>S. olivaceus</i>	B-99	5	61	61	60	62	66	67	67	67	67	63	64	66	71	71	66	66	61	55	57	57	58	58	62	62	63	63	62	98	98	98	98	100																				
		16	18	17	12	27	31	26	11	20	15	30	29	32	28	14	13	19	25	22	24	23	21	6	9	8	7	10	3	2	4	1	5																					

colour group. The differences were expressed with the numbers of the Color Harmony Manual chips on Tresner—Backus's colour wheel proposed for the definition of fine but characteristic hues of the colour of the sporulating aerial mycelium of actinomycetes (e265-medium gray; 3fe-light brownish gray; 5ih-brownish gray).

The variability of predominant intestinal *Streptomyces* species is demonstrated in Table IV, where the representative strains are grouped according to percentage similarity computed on the basis of 84 properties. The strains, which had been isolated from the larvae of 6 *Bibio* populations, formed 4 distinct groups (1 to 4) corresponding to the above-mentioned 4 species. The distinct systematic position of three groups (species) is indicated by similarity values higher than 97, 96, and 95 per cent for *S. olivaceus*, *S. antibioticus* and *S. aureofaciens* strains isolated from larval populations 1, 2, and 5, respectively. *S. finlayi*, as in the previous study, comprised a large but loose group, the members of which were linked with similarity values ranging from 79 to 99 per cent. *S. finlayi* strains were not divided by the computer into 3 subgroups corresponding to the 3 different larval populations in which they were predominating, but, on the basis of the average similarity, arranged in a sequence not associated with their origin. Similarly to previous findings [1], strains with hairy and smooth spore surface were placed separately (subgroups "1a" and "1b"). Thus *S. finlayi* strains, which were characterized by a uniform green substrate mycel pigmentation, showed heterogeneous physiological and morphological properties independently of the larval populations they had been isolated from. The two variants, which differed primarily in spore surface morphology, occurred always together. The organisms showed a high degree of variability in salt tolerance and antibiotic sensitivity. On the basis of a large number of stable + or - characters, however, these strains undoubtedly belonged to one species. Finally, in the previous as well as in the present studies strains 4161, 4174, 4162, 4169, 4167, 4200 and 4165 were included in the numeric taxonomical analysis of *S. finlayi* strains. Similarity values in Table IV for each pair of these strains were always some per cent higher than the previously obtained corresponding values. This finding may be attributed to the fact that in the present study a number of previously used characters were omitted and replaced by others. A further factor was that the number of considered properties was increased from 76 to 84.

Examination of the intestinal actinomycetes flora of individual larvae yielded, with some unimportant individual differences, the results obtained with pooled (mixed) gut content samples of the *Bibio* populations.

General ecological-physiological description of intestinal actinomycetes flora. Strains of the predominant species are unable to grow at 40°C or higher, do not utilize cellulose as a sole source of carbon, do not fix atmospheric nitrogen, grow in the presence of 20 per cent $MgSO_4$, are resistant to penicillin and

polymyxin-B, do not produce inhibitory substances against Gram negative organisms, usually decompose wax, grow abundantly with dextrin, do not utilize salicin and usually do not grow with sucrose. In a presumable humification in the alimentary canal only the chromogenic *S. antibioticus* may have had a role. Some strains decomposed paraffins. *S. olivaceus* strains were, with exceptions, unable to utilize NaNO_3 . The consistent failure to reduce nitrates, or more exactly a negative nitrite reaction, was observed with *S. antibioticus* strains only. The predominant streptomycetes of larval populations 1 and 2 were inactive against Gram positive and Gram negative test organisms and *Saccharomyces carlsbergiensis*. Intestinal multiplication of these organisms was not associated with their antibiotic activity on intestinal bacteria. In antibiotic sensitivity the strains of species *S. olivaceus*, *antibioticus*, and *aureofaciens* were less variable while *S. finlayi* cultures showed considerable differences. *S. olivaceus* cultures were especially sensitive to sulphonamides, streptomycin, chloramphenicol and aureomycin. A group of *S. finlayi* strains was resistant to sulphonamides, chloramphenicol, erythromycin, aureomycin, terramycin, etc. Most of the strains of the *Streptomyces* species which were common in the gut of *Bibio* larvae showed a marked susceptibility to different antibiotics.

Discussion

In a previous paper [1] it was shown that the soil actinomycetes flora containing the complex population of numerous different — partly very common — species is subjected to radical selection during intestinal passage and, in consequence of this alteration of species composition, one species, namely *S. finlayi*, became absolutely predominant in the gut actinomycetes flora of the larvae. It remained questionable whether the predominance of this organism is stable and typical and whether it was justified to assume the presence of a specific and stable intestinal actinomycetes flora in the gut of the larvae.

The present examinations indicate that *S. finlayi* displays a considerable affinity to the intestinal milieu of the larvae, since it was predominating in the gut of larvae of other two investigated *Bibio* populations. In the larvae of three populations, however, the absolute predominance of other — systematically sharply distinguishable — species was observed. This finding confirms only the mono- or oligospecies character of the intestinal actinomycetes flora but not its stable specificity. The soil under the activity area of all the examined larval populations was forest mull-like rendzina. Some species, which occurred in large numbers in all the samples taken from horizons A_H and A_F (*S. chartreusis*, *Noc. alba*, etc.) were absent from the intestine. Not all soil actinomycetes seem, therefore, to have an equal chance of becoming predominant "intestinal organisms". Had the isolated species been arranged in

respect to capacity of intestinal selection, *S. finlayi*, this extraordinarily variable and ecologically probably highly plastic organism, would occupy the first place while *S. chartreusis* would be among the species falling at the end of the row.

In other investigations we have shown that 2 or 3 actinomycetes species may occur simultaneously in high proportions (30 to 40 per cent) in the intestine of *Bibio* larvae.

From a comparison of the actinomycetes flora in the gut of *Bibio* larvae belonging to one population and in the soil these animals are feeding on, it would appear that the intestinal environment is characterized by a specific microbial association. However, if other larval populations are taken into consideration, it becomes evident that this specificity — similarly to the organization of all microbial communities in soil or on root surface [27] — is only a relative one.

A further interesting problem is the usefulness of computer analysis in the classification of actinomycetes. This method has been criticized by several authors and its use has not yet been suggested by the *Subcommittee on Streptomyces of the International Committee on Bacteriological Nomenclature*, the international coordinator of taxonomic studies on actinomycetes. Our results indicate that similarity data computed on the basis of 70 to 100 characters mean a great help in the identification of related strain-groups or species and in the estimation of the specificity of microbial associations. In drawing the borderline between species, variants or genera at a consistently identical similarity level, much caution is recommended. As seen from the examples presented in this study, the populations of the individual species may show very different degree of variation.

In a subsequent paper a general ecological-physiological description of enteric bacteria and a comparative computer analysis of intestinal and soil bacterium flora will be presented.

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EFFECT OF MILK FROM COWS WITH MASTITIS ON THE ACTIVITY OF BUTTER CULTURE

By

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(Received November 24, 1966)

Summary. Formation of lactic acid and aroma by, and physical properties of, bacterial cultures used in butter production have been examined in raw and heated milk samples selected by the Whiteside test. In final acidity and total bacterial count, cultures grown in milk obtained from cows with mastitis were considerably lower than normal milk cultures. Cultures made in pathologic samples were flavourless with a strange bitter, nauseating taste and were characterized by a soft, flaky clot expelling whey. In milk of cows with mastitis, probably because of a lower buffer capacity, the growth of *Streptococcus lactis*, *Streptococcus cremoris* and *Leuconostoc* was inhibited. The inhibitory effect was presumably promoted by the presence of heat labile (sensitive to 85° C) and heat stable (resistant to sterilization for 30 minutes) antibodies and partial deficiency in bios factors.

As estimated by acid content determination, the considerable inhibitory effect was not demonstrable when normal milk contained less than 5 per cent pathologic milk. Production of aroma by, and the taste and consistency of, the culture were influenced by smaller proportions of pathologic milk.

In view of its incidence, the danger to the community and economic loss, mastitis constitutes the most important pathological condition of the udder.

In Hungary, as well as in many other countries, mastitis is a widespread disease [1, 2, 18]. It has been found that while the incidence of acute mastitis is 0 to 30 per cent, that of subclinical mastitis is 70 to 100 per cent. Sever acute inflammation causes no diagnostic difficulty and the milk of such animals is usually separated from healthy cows' milk. In contrast, in subclinical, chronic mastitis there are no symptoms and changes in the physical properties of milk, although the causative agent and an alteration in chemical and biological properties, similar to those in acute inflammation, can be demonstrated. Milk from cows with chronic mastitis is usually mixed with normal milk and employed for industrial purposes.

The good quality of the end product depends on the quality of the bacterial culture used in its preparation. A proper butter culture produces sufficient acidity, is free from disagreeable odour and taste and gives at least a ++ aroma reaction. It forms a smooth clot and produces usually an acid content of 36 SH°. Inadequate clotting, failures in odour, taste and consistence are mostly caused by alterations in the milk. Hence, it was of interest to study the effect of milk originating from cows suffering from mastitis on lactic acid and aroma production, and the physical properties of the culture.

Materials and methods

Milk samples were selected by means of the Whiteside test [11, 12]. Acid content of milk and butter culture was determined by the Soxhlet—Henkel method, the pH by a pH-meter.

Aroma content of the butter culture was estimated in parallel determinations by the technique of VAS and CSISZÁR [4] and by RICHTER's modification of the Voges—Proskauer method [5]. The degree of the reaction was expressed as +, ++, +++, ++++ and — (negative). As the two methods gave closely similar results, the data obtained with VAS and CSISZÁR's technique are only demonstrated.

The microscopic picture of milk and culture was examined in smears stained with toluidine blue.

Results

1. Lactic acid and aroma production in raw and heated pathologic milk.

Whiteside negative (normal) and positive (pathologic) samples were collected separately under the strictest hygienic precautions attainable. Care was exercised to obtain positive samples only from cows with subclinical symptoms, that is, samples with no visible alterations. Mixed milk from cows with mastitis gave a +++ Whiteside reaction. Milk specimens from healthy cows were always negative. The cows received no antibiotic therapy during the experiments.

Raw milk and samples heated at 75, 85 and 100° C for 1 minute or sterilized at 1.2 at pressure for 30 minutes were seeded with 0.5 per cent butter culture. Culturing was performed at 22° C for 24 hours. The rate of acid production in negative and positive milk and bacterial counts after 24 hours incubation is presented in Table I.

It is seen that acid production in negative milk showed an adequate course, while in positive milk the latency period was somewhat prolonged and acid production was slower. The final acid content of raw and heated normal milk was 37.7 to 41.2 SH°. The acid content of positive samples was usually 11 to 14 SH° less and never exceeded 30 SH°. The final acid content of negative and positive samples, which had been heated at 75° C, was lower than that of samples exposed to higher temperatures. With the increase of temperature the cultures' acid content increased more markedly in positive than in negative samples.

Mean final acidity was proportional to the average of final bacterial count.

Negative milk samples, with the exception of raw ones, formed clot in 10 hours; raw samples showed after 10 hours partial, after 12 hours, total clotting. In consequence of a lower multiplication rate and a slower acid production, pathologic milk samples, which had been heated at 85° and 100° C or sterilized, formed clot only in 24 hours. Pasteurized (75° C) samples had shown a weak clotting by this time, while raw samples formed no clot at all.

Table I

Acid production by butter culture in Whiteside negative and positive milk samples subjected to different heat treatments

	Negative milk					Positive milk				
	Raw	75°	85°	100°	Auto-clave	Raw	75°	85°	100°	Auto-clave
Acid content (SH°)										
Initial	7.1	6.7	6.7	6.7	6.7	4.1	3.8	3.8	3.8	3.8
2 hours	7.4	7.3	7.2	7.2	7.3	4.3	4.1	4.3	4.2	4.3
4 hours	8.3	8.8	8.6	8.8	9.5	5.4	5.2	5.3	5.1	5.5
6 hours	10.6	13.8	13.2	13.8	14.7	6.9	7.7	8.6	7.8	8.2
8 hours	20.5	24.6	29.7	23.5	24.3	12.5	16.0	19.9	17.0	20.1
10 hours	26.9	30.1	34.6	30.9	31.5	16.8	19.5	22.9	23.2	24.2
24 hours	38.2	37.7	41.2	40.7	39.2	23.8	25.5	28.5	27.6	28.0
Difference in SH°						-14.4	-12.2	-12.7	-13.1	-11.2
Total bacterial count, million/ml, after 24 hours	7200	5920	12000	8100	6830	860	910	2700	2270	2620

The clot formed in normal milk was homogeneous, smooth, characteristic of lactic acid fermentation, and gave a ++ aroma reaction. In pathologic samples the clot was soft, flaky, layered, sedimental. Many of the latter samples showed a slight shrinking and gas bubbles. The aroma content was practically nil.

The microscopic picture of normal milk cultures was characterized by well-developed cocci (mainly diplococci and tetrads) and small numbers of leucocytes. In pathologic milk, less developed and degenerated cocci (mainly in chains) prevailed and leucocytes occurred in high numbers.

2. *Effect of buffer capacity of pathologic milk on the biological activity of butter culture.* The increase in acidity was similar in negative and positive milk until 17 to 24 SH° had been reached; the only difference was that in positive samples the latency period was longer and the rate of acid production slower. The acid content of negative samples then increased up to 38–41 SH°; that of positive samples remained under 30 SH°. This has been attributed, at least partly, to the low buffer capacity of pathologic milk.

Buffer capacity was estimated by comparing the acid content and pH of raw and heated samples. The effect of higher leucocyte count on the multiplication of lactic acid bacteria and pH of the sample was also considered.

After estimating the original acid content and pH, negative, positive and centrifuged positive samples were inoculated with 0.5 per cent butter culture. The positive samples gave a +++ Whiteside reaction. After centrifugation the supernatant of the positive samples became Whiteside negative and its leucocyte count decreased from the original 5 million to 250,000—500,000. The samples were cultured at 22°C and their acid content and pH were determined after 2, 5, 8, 10 and 24 hours incubation.

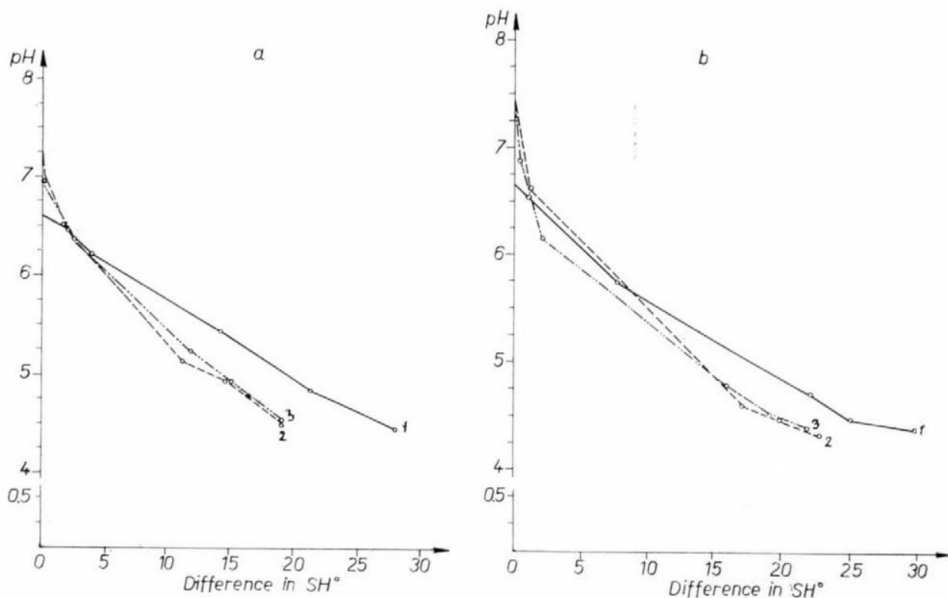


Fig. 1. Relationship between pH and acid content of Whiteside negative (1), positive, non-centrifuged (2), and positive centrifuged (3), milk seeded with butter culture. a = raw milk, b = sterilized milk

The pH of the samples plotted against the difference in SH° is presented in Fig. 1.

It is seen that the decrease of pH was more rapid in positive and centrifuged positive than in negative milk, although acid production was slower in the former two samples.

The original pH of negative milk was 6.6, of positive milk 7.3. After 24 hours incubation the pH of negative raw and sterilized samples was 4.45 and 4.40, of positive raw and sterilized samples 4.50 and 4.35, of centrifuged positive raw and sterilized samples 4.53 and 4.41, respectively. A 28.0 SH° acid content in negative raw milk lowered the pH by 2.15, a 29.7 SH° acid content in negative sterilized milk, by 2.20. In positive raw and sterilized milk the increase in acid content amounting to 17.3 and 21.6 SH° was associated

with pH values 2.10 and 2.25 units lower than the original pH 6.6. The corresponding values in centrifuged positive raw and sterilized milk were pH 2.07 and 2.19 for 17.5 and 21.5 SH° increase, respectively.

3. *Lactic acid and aroma production in samples containing pathologic and normal milk.* It was desirable to examine the amount of positive milk which could be added to negative milk without causing an inhibition of the activity of butter culture. Negative and positive (nearly +++) samples and their mixtures containing 50, 40, 30, 20, 10, 5, 2.5, 1, 0.5 and 0.1 per cent positive milk were examined for acid content, aroma, taste and consistence. The samples were pasteurized at 85° C for 1 minute, then inoculated and incubated as in previous experiments. Table II demonstrates the increase in acid and aroma content.

The increase in acid content was less in positive than in negative milk. With decreasing the proportion of pathologic milk there was a gradual increase in final acid content. The inhibitory effect was still definite at 10 per cent, but was not demonstrable at 5 per cent positive milk content.

The negative sample gave a ++, the positive a — aroma reaction. In a mixture containing 2.5 per cent positive milk the reaction was less than +; 1 to 0.5 per cent positive milk content still influenced the aroma (+ reaction). The ++ reaction characteristic of pure negative milk was obtained only in mixtures containing not more than 0.1 per cent positive milk. It can therefore be concluded that aroma-producing organisms of the butter culture are highly sensitive to the presence of pathologic milk, or due to a change in composition, positive milk is unsuitable for aroma production.

The taste of culture grown in normal milk was aromatic, sour and pleasant, that grown in pathologic milk was strongly acrid, bitter, nauseating and unpleasant. No bitter taste was produced when less than 5 per cent positive milk was incorporated, but the acrid taste disappeared only under 2.5 per cent pathologic milk content. In the presence of 1, 0.5 and 0.1 per cent positive milk the taste was similar to that of negative milk cultures. Thus even 2.5 per cent positive milk may cause an unpleasant taste. Because of the said properties the culture prepared from pathologic milk is unsuitable for use.

The culture prepared from negative milk was homogeneous and smooth. It was characterized by typical lactic acid fermentation with no expelling of the whey. In contrast, pathologic milk showed only partial clotting after 24 hours. The clot of samples containing 50 to 40 per cent positive milk was soft, flaky, of those containing 30 to 2.5 per cent positive milk was slightly flaky with many gas bubbles. Only samples in which 1, 0.5 or 0.1 per cent positive milk had been incorporated, clotted similarly to negative milk.

Table II

Acid and aroma production by butter culture in Whiteside negative and positive milk samples mixed in different proportions

	Negative sample	Positive sample	Mixed samples (percentage negative + positive)									
			50 + 50	60 + 40	70 + 30	80 + 20	90 + 10	95 + 5	97.5 + 2.5	99 + 1	99.5 + 0.5	99.9 + 0.1
Acid content (SH°)												
Initial	7.0	3.8	5.4	5.6	6.4	6.6	6.8	6.9	7.0	7.0	7.0	7.0
2 hours	7.4	3.8	5.4	5.8	6.8	6.8	7.0	7.0	7.2	7.2	7.2	7.3
4 hours	8.4	4.4	6.6	7.2	8.1	8.2	8.2	8.5	8.4	8.4	8.6	8.4
6 hours	13.2	6.4	9.6	10.5	10.3	11.1	11.5	11.5	12.0	12.8	12.5	12.9
8 hours	28.8	13.0	23.6	24.7	26.4	26.8	26.2	28.1	28.1	28.8	28.8	29.6
10 hours	32.0	23.0	26.7	28.4	30.0	30.0	30.2	31.2	32.0	32.6	32.6	32.0
24 hours	39.6	28.8	32.8	34.3	35.6	36.4	36.7	39.6	39.8	39.2	39.8	38.8
Difference in SH°	—	—10.8	—6.8	—5.3	—4.0	—3.2	—2.9	0.0	+0.2	—0.4	+0.2	—0.8
Aroma reaction	++	—	—	—	—	—	—	±	±	+	+	++

Discussion

It has been shown that acid production by organisms of the butter culture in milk of cows with mastitis is slower, and the final acid content is 11 to 14 SH° less than in normal milk cultures. The bacterial count of cultures grown in raw or heated negative milk is 3 to 9 times higher than of those prepared in positive samples. The considerable difference in final acid content and bacterial count is probably due to the fact that pathologic milk inhibits the multiplication of lactic acid bacteria. This inhibitory effect was demonstrated by many authors. PETERSEN [14], NYIREDY [13], McDOWALL [9, 10] and SINGH [20] attributed the phenomenon to "antibacterial factors". DAVIS and McCLEMONT [3] concluded that decrease in acid production in pathologic milk was due to the lack of certain growth factors.

The high difference in final acid content of raw and heated positive milk (Table I) indicates that the sample contained higher amounts of thermolabile antibodies (sensitive to 85°C) than did the negative samples. Since the final acid content was much less in positive samples than after exposure to higher temperatures, it may be concluded that during mastitis highly thermoresistant inhibitory substances appear in milk. These factors may be produced by the causative organism.

In order to show whether the inhibitory effect was or was not associated with infection, the following experiments were performed. (1) To enhance multiplication of the causative agent, positive milk was incubated at 27°C for 24 hours; (2) aliquots of normal milk were seeded with pure cultures of *Str. agalactiae*, *Str. dysgalactiae*, *Str. uberis* and *Staphylococcus aureus*; incubation lasted for 12 hours. The cultures were then heated as described above and finally inoculated with butter culture. The same Whiteside negative milk served as control. There was no significant difference in acid content of the samples.

Detection of inhibitory substances in the supercentrifuged deposit and supernatant of the above samples was attempted by means of the agar diffusion technique. Neither positive, nor negative samples inoculated with the mentioned pathogens produced any zone of inhibition.

It is therefore probable that metabolic products of the causative agent cannot be responsible for the decrease of acid production. The phenomenon seems to be associated with a change in the constituents of the milk or to other factors such as thermolabile and thermostable antibodies. For the lower rate of acid production the partial lack of bios factors may also be responsible.

Fig. 1 indicates that the final pH was practically the same in negative and centrifuged and noncentrifuged positive milk cultures, despite the considerable difference in acid content. As compared to negative milk, in positive samples 2/3 of the amount of lactic acid produced the same reduction in pH.

This finding indicates that pathologic milk exhibits a decreased buffer capacity, as confirmed by the observation that in positive milk, similarly to negative samples, multiplication of lactic acid bacteria discontinues at pH 4.4; however, when growth had stopped, positive samples were found to contain 11–14 SH° less acid than negative milk cultures.

WHITEHEAD [17] attributed the inhibition of growth to the high leukocyte content of positive milk (over 5 million/ml). In this study no difference was demonstrated in acid production and even buffer capacity between centrifuged and non-centrifuged positive milk.

Aroma production by *Leuconostoc citrovorum* and *Leuc. dextranicum* proceeds best at pH 4.1 to 4.4 [15]. The final pH of milk seeded with butter culture falls in this range (sterilized positive milk), yet, the culture grown in pathologic milk gave a negative or at most $\pm$ aroma reaction in contrast to the $++$ reaction of normal milk culture. This finding indicates that pathologic milk contains some substances which are responsible not only for the lower buffer capacity but also for the inhibitory effect on butter culture. The phenomenon is probably a resultant of the different factors mentioned.

The presence of 5 per cent positive milk caused no growth inhibition or decreased acid production. However, aroma, taste and consistence of the culture were disadvantageously influenced by much lower concentrations of positive milk.

The lowest concentration of pathologic milk in normal milk exerting inhibitory effect is 1 per cent according to NYIREDY [13], 1 to 20 per cent according to McDOWALL [10] and 5 to 10 per cent according to KOZHEVNIKOV [6]. The differences are due to the use of milk samples obtained from cows with different clinical forms of mastitis. NYIREDY examined milk samples from animals with manifest mastitis, while McDOWALL used milk from cows suffering from acute as well as from latent infection. Milk of cows with acute mastitis is not being used for industrial production. Therefore the harmful effect of milk of cows with subclinical mastitis bears a great industrial importance.

Average mixed milk may be estimated to contain 5 to 40 per cent Whiteside positive milk, and thus presents a constant danger for the quality of dairy products prepared by use of butter culture. Such milk samples should accordingly be excluded from industrial production by individual selection. Our experiments have shown that the Whiteside test is a suitable rapid method for this purpose.

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ANTIGENIC STRUCTURE OF MYCOBACTERIUM KANSASII AND ITS VARIANTS

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(Received December 1, 1966)

Summary. Immune-diffusion, immune-electrophoresis and immune-absorption tests have been performed with 12 *M. kansasii* strains including scotochromogenic and albino variants. Independently of pigment production all strains were uniform in antigenic structure. The studies were supplemented with bacteriological and biochemical examinations.

It has been shown that *M. kansasii* is well characterized by biochemical and immunological properties. RUNYON's classification based on pigment production is insufficient for the determination of atypical mycobacteria. In addition to biochemical properties, antigenic structure is considered the main basis of classification.

Among "anonymous" acid-fast bacteria described in recent years, the photochromogenic organism isolated by POLLAK and BUHLER [1] is especially important. On the basis of pigment production RUNYON [2] classified this bacterium into group I of atypical strains. HAUDUROY [3] recommended the name *Mycobacterium kansasii*. These bacteria deserve interest not only because of their possible pathogenicity but also because of their variability in pigment production constituting the basis of classification. The organism may produce scotochromogenic (RUNYON's group II) and even non-pigmented (albino) variants [4—9].

CASTELNUOVO and MORELLINI showed that *M. kansasii* luciflavum strains were antigenically related [10]. In contrast, NORLIN revealed serological difference in these strains [11]. The contradictory data and the possible variability of mycobacteria, which is not merely a theoretical, but an important diagnostic problem, indicated the necessity for further studies. In the present paper an account is given of the comparative serological analysis of strains obtained from different laboratories. In order to show the usefulness of serological methods in classification, the data are compared with bacteriological and biochemical properties characteristic of the species.

Materials and methods

Strains. *M. kansasii* strains 232, 226, 2537, 2438, 2504 and 2437 were received from the International Centre for Information on, and Distribution of, Type Cultures, Lausanne. Strains 1250 B 1, T 109 A, T 109 B, Czerw A and Czerw B were obtained from the Institut Pasteur, Lille, and strain "*M. kansasii*" from the Korányi National Institute for Tuberculosis, Budapest.

Bacteriological methods. (a) Growth on Löwenstein-Jensen and in Sula medium at room temperature, 37°C and 45°C. (b) Proportional indirect antibiogram as described by CANETTI *et al.* [12]. (c) Pigment production in dark and 18 hours after 1 hour exposure to a 60 W electric bulb.

Biochemical properties. (a) Niacin, catalase and peroxidase reactions. (b) Lipase activity as tested by TISON *et al.* [13]. (c) BÖNICKE's amide reactions [14].

Immunological methods. (a) Immune diffusion by OUCHTERLONY's method [15]. (b) Immune electrophoresis by GRABAR and BURTIN's [16] modified method [17]. (c) Preparation of immune sera: two week Sauton cultures of strains 232, 226 and 2437 were treated with 1 per cent phenol for 12 hours, then filtered and washed with sterile distilled water. The wet bacteria were suspended in a mixture of 85 per cent Bayol "5" liquid paraffin and 15 per cent Arlacel "A" so that the final concentration of bacteria was 100 mg/ml. Rabbits weighing 2.2 to 2.8 kg were immunized with multiplex techniques intracutaneously and subcutaneously with 6 to 8 weekly doses of 1 ml suspension. Before injection the oily suspension was mixed with one part of saline. Ten to 14 days after the last injection the rabbits were bled. The serum was passed through Seitz EK filter and stored at -10°C. (d) Antigen solution was prepared by GIMPL's method [17] from 6 to 8 week Sauton cultures. (e) Immune-absorption experiments were performed by mixing the immune sera with equal parts of antigen solutions of strains 232, 2437 and 1250 B 1, refrigerating them at 4°C overnight then centrifuging at 6000 r. p. m. The supernatant was used as absorbed immune serum.

Results

M. kansasii strains grew slowly at room temperature. No multiplication was observed at 45°C. At the optimal 37°C they grew in 7 to 10 days. Proportional indirect antibiotic sensitivity testing showed that the strains were resistant to isonicid, streptomycin and PAS and sensitive to ethionamide, cycloserine, viomycin and resistomycin. Of the strains 8 were photochromogenic, 3 scotochromogenic and 1 non-chromogenic. Independently of pigment production, all strains were niacin negative, catalase and peroxidase positive, showed high lipase activity, exhibited a characteristic amide spectrum, decomposed urea and nicotinamide (Table I).

With immune diffusion and immune electrophoresis in homologous and heterologous immune-precipitation systems, 7 to 10 antigenic components were usually detected. Examination in anti-photochromogenic and anti-scotochromogenic sera indicated that all strains were identical antigenically. (Figures 1, 2 and 3). Although the data presented were averaged from the results of several experiments, small differences in the number of components do not disprove the antigenic identity of the strains. The differences may have been due to the fact that some components were present in variable amounts in the filtrate of the culture medium and accordingly they were not always present in proper equivalence needed for precipitation. The finding, however, that no precipitation lines were observed after the absorption of the sera with photo, scoto or non-chromogenic strains, confirms that *M. kansasii* is uniform in antigenic structure regardless of pigment production.

Table I
Pigment production by, and biochemical properties of, M. kansasii strains

Strain	Pigment production	Niacin	Catalase	Peroxidase	Lipase	Acetamide	Benzamide	Urea	Isonicotinamide	Nicotinamide	Pyrazinamide	Salicylamide	Allantoin	Succinamide	Malonamide
232	Photochromogenic	—	+++	+	+	—	—	+	—	+	—	—	—	—	—
2437	Scotochromogenic	—	+++	+	+	—	—	+	—	+	—	—	—	—	—
1250 B 1	Non-chromogenic	—	+++	+	+	—	—	+	—	+	—	—	—	—	—
226	Photochromogenic	—	+++	+	+	—	—	+	—	+	—	—	—	—	—
2537	Photochromogenic	—	+++	+	+	—	—	+	—	+	—	—	—	—	—
2438	Photochromogenic	—	+++	+	+	—	—	+	—	+	—	—	—	—	—
T 109 A	Photochromogenic	—	+++	+	+	—	—	+	—	+	—	—	—	—	—
T 109 B	Scotochromogenic	—	+++	+	+	—	—	+	—	+	—	—	—	—	—
Czerw A	Photochromogenic	—	+++	+	+	—	—	+	—	+	—	—	—	—	—
Czerw B	Scotochromogenic	—	+++	+	+	—	—	+	—	+	—	—	—	—	—
<i>M. kansasii</i>	Photochromogenic	—	+++	+	+	—	—	+	—	+	—	—	—	—	—
2504	Photochromogenic	—	+++	+	+	—	—	+	—	+	—	—	—	—	—

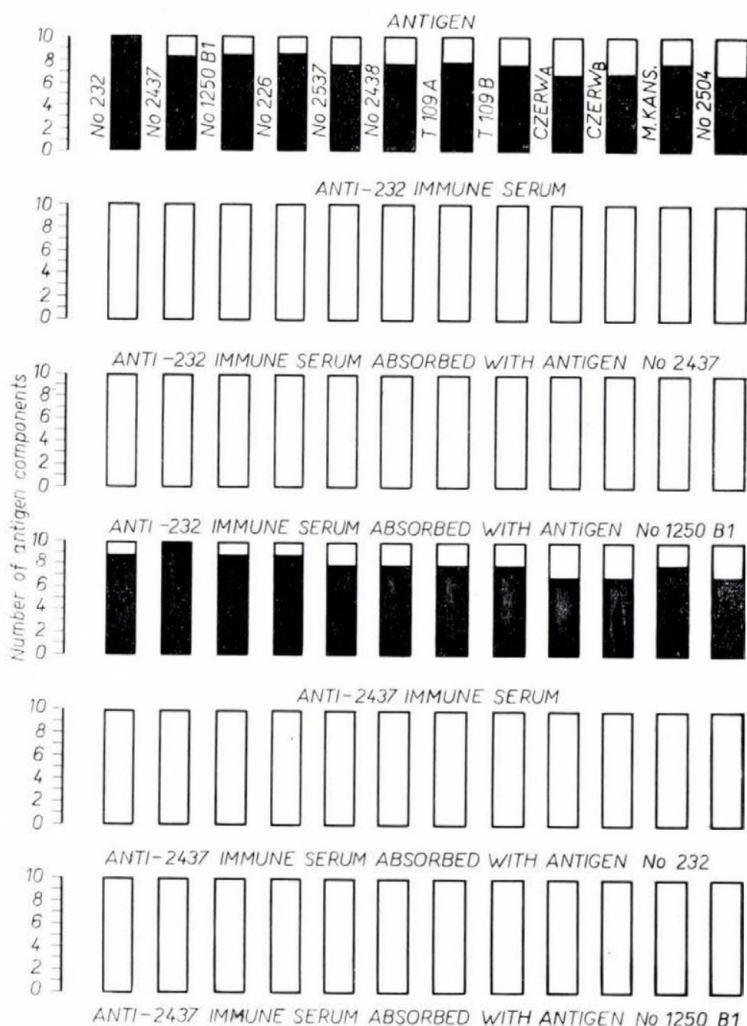


Fig. 1. Minimum number of antigenic components of *M. kansasii* strains detected by immune diffusion, immune electrophoresis and immune absorption

Discussion

M. kansasii strains originating from different laboratories have been examined. In bacteriological, biochemical and immunological properties the strains were uniform and identical with the original POLLAK—BUHLER strains (cultures 232 and 226). Variation in pigment production did not affect the basic biochemical and serological properties of the strains. It may be concluded that *M. kansasii*, independently of the source and pigment production of its strains

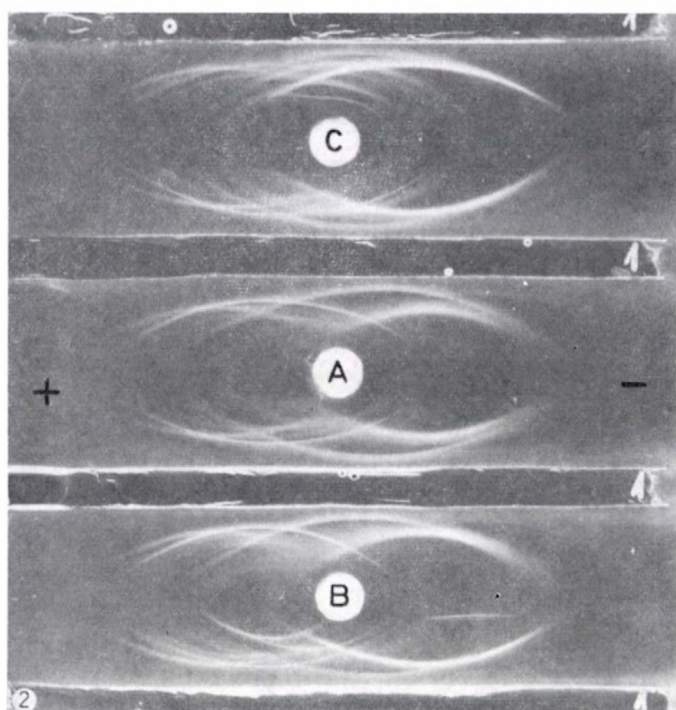


Fig. 2. Immune electrophoresis of antigen solutions 232, 2437 and 1250 B 1 with anti-232 immune serum. Well "A": 232; well "B": 1250 B 1; well "C": 2437; trough "1": anti-232 serum

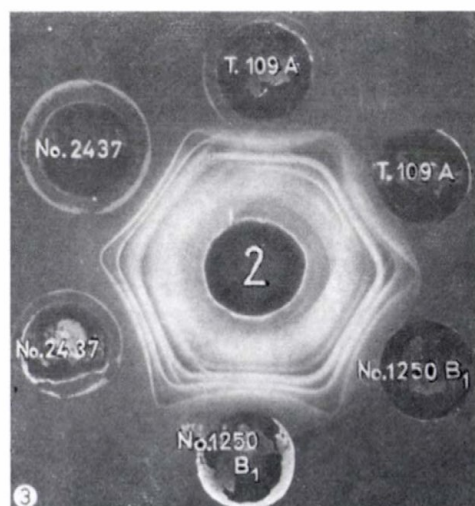


Fig. 3. Immune diffusion of antigen solutions 2437, 1250 B 1 and T 109 A with anti-2437 immune serum. Well "2" contains anti-2437 serum

is a well-defined uniform species. RUNYON's classification based on pigment production is insufficient for taxonomic purposes. In the classification of atypical mycobacteria, in addition to biochemical behaviour, serological properties constitute the main differential criterion.

Acknowledgement. We are indebted to *Professor P. HAUDUROY* (Lausanne), *Professor A. TACQUET* (Lille) and *DR. I. SZABÓ* (Budapest) for supplying the strains.

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THE ANTIGENIC STRUCTURE OF RAT SARCOMA

By

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(Received December 10, 1966)

Summary. In the serum of rabbits immunized with Benevolenskaya rat sarcoma extract several antibodies reacting with various rat plasma proteins have been shown by OUCHTERLONY's radial double immune diffusion method. Antitumour sera prepared in rabbits immunologically tolerant to rat plasma reacted only with one component of rat plasma and with four components of tumour extract. Each of these four components was present in the liver and spleen; three of them were demonstrated in the kidney and small intestine and two of them in the lung; one, split into two parts, was present in the heart muscle; none of the components was shown in striated muscle. No antigen specific for the tumour was revealed.

Immune diffusion methods have successfully been applied in immunological studies on the antigenic structure of organs and tumours. Methods have been described for the identification of enzymes and nucleoproteins in tissue extracts [1–5]. In studies of the antigenic structure, masking of tissue antigens by plasma proteins presents considerable difficulties. BJÖRKLUND [6, 7] has shown that antibodies to cellular antigens appear only after a prolonged immunization period. As in anti-rabbit bone marrow or anti-human tumour horse sera prepared in this manner several serum components react with normal rabbit or human plasma, evaluation of OUCHTERLONY's test is rather difficult. From the preliminary experiments mentioned below similar conclusions have been drawn. In recent years it has been described that immunological tolerance can be brought about with antigen mixtures, namely with tissue antigens [8, 9] and whole blood plasma [10]. These findings have made it possible to extend the studies on the antigenic structure of organs and tumours.

Materials and methods

The experiments were carried out with Benevolenskaya sarcoma maintained by serial transfer in white rats [11]. The antigen for immunization was prepared as follows. The tumour was separated from the neighbouring tissues and necrotic parts of the tumour, homogenized in a Turmix blender (1 g wet weight in 2 ml saline). The homogenate was preserved with 0.1 per cent merthiolate + 0.1 per cent streptomycin and absorbed to aluminium hydroxide gel.

In preliminary experiments 2 rabbits weighing 2.5 kg each were injected intramuscularly with 0.7 g wet weight tumour doses at weekly intervals over a period of 13 weeks. The animals were bled 2 weeks after the last injection. The sera were preserved with 0.1 per cent merthiolate + 0.1 per cent streptomycin and stored in the deep freeze.

GARB and STEIN [10] succeeded in inducing tolerance to plasma proteins in newborn rabbits by injecting whole blood. On the basis of this observation in the present experiments

each of 8 newborn rabbits was injected subcutaneously with 0.5 ml citrated rat blood within 6 hours after birth. The same dose was repeated weekly over a 2 month, then at three week intervals over a 5 month, period. For some unknown reason, three animals died a few days after birth. The fourth was lost during the second month. From the remaining 4 rabbits blood samples were taken 2 weeks after the last injection. In the tube precipitation test with rat plasma two of the sera failed to react; the other two sera gave 1 : 10 titre precipitation. The two negative rabbits were then immunized with 13 injections of tumour antigen given at 7 to 10 day intervals. The same doses were used as in the preliminary experiments. The animals were bled 2 weeks after the last injection. The sera were preserved as described above and stored in the deep freeze.

Gel diffusion tests were performed with OUCHTERLONY's original technique [12], BJÖRKLUND's inhibition technique [6] and our own modified method [13]. One per cent Difco agar dissolved in physiological saline was used throughout the experiments. Tissue extracts for the immune diffusion test were prepared as follows. One g of tissue was homogenized with 2 ml saline in the Potter glass homogenizer. After centrifugation at 3000 r. p. m. for 15 minutes the supernatant was pipetted off and used as an antigen. Each gel diffusion experiment was repeated at least 5 times. Immune diffusion plates were photographed as described by ALMEIDA *et al.* [14].

Results

Preliminary experiments showed that an anti-tumour serum prepared in rabbits not tolerant to rat plasma gave a strong reaction with many components of rat plasma. This phenomenon made it difficult to evaluate the reactions given by tissue antigens. Fig. 1 indicates that the tumour extract

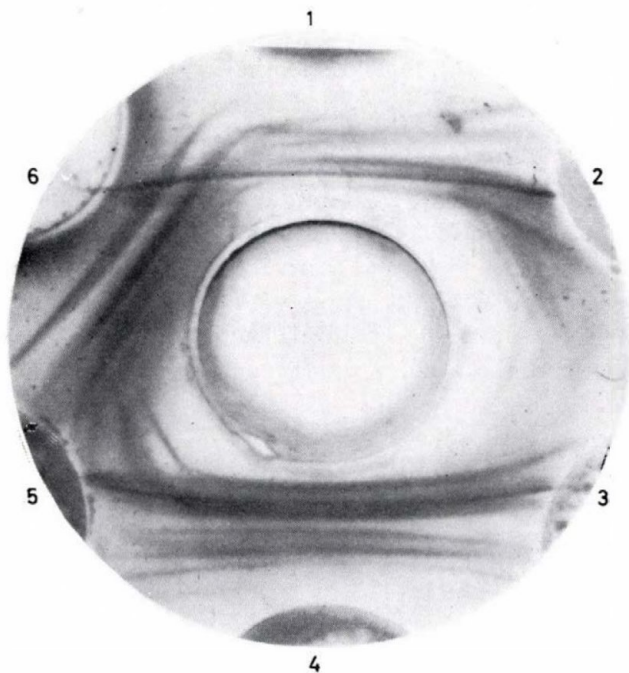


Fig. 1. Original Ouchterlony plate. Centre well: antitumour serum prepared in non-tolerant rabbit. Antigens: 1,4 = tumour, 2 = kidney, 3 = intestine, 5 = muscle, 6 = plasma

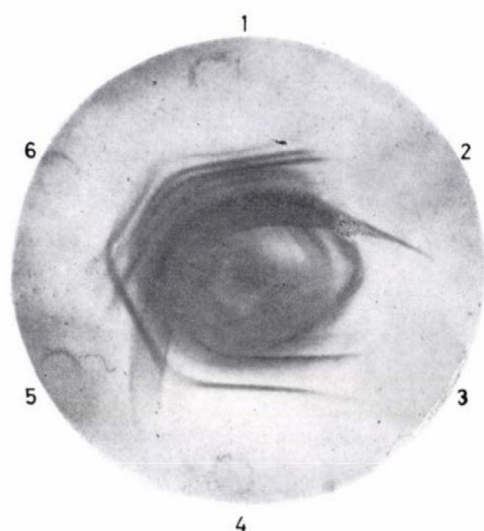


Fig. 2. Modified Ouchterlony plate. Centre well: see Fig. 1. Antigens: 1,6 = tumour, 2,3 = plasma, 4,5 = liver

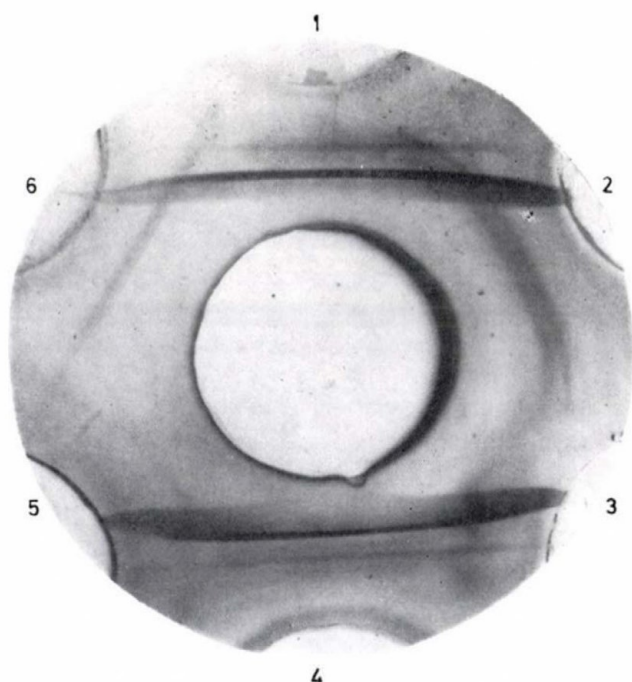


Fig. 3. Björklund inhibition plate. Centre well: see Fig. 1. Antigens: 1,4 = tumour, 2 = kidney, 3 = intestine, 5 = muscle, 6 = plasma

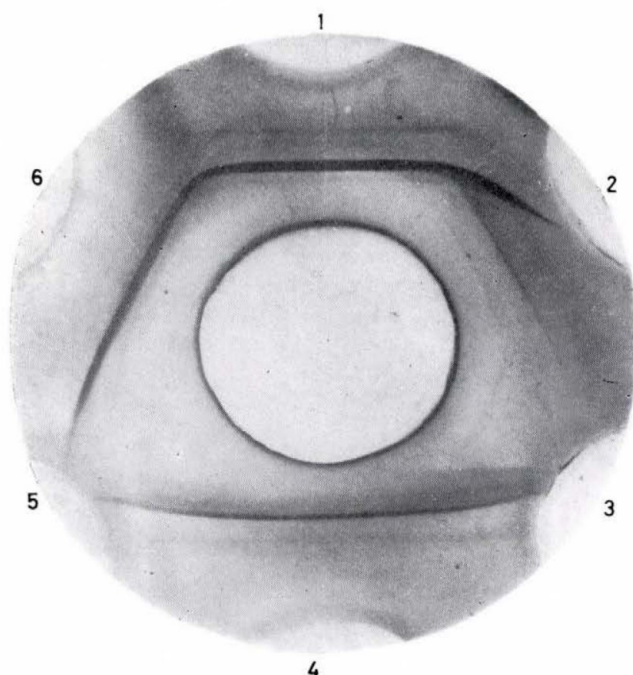


Fig. 4. Björklund inhibition plate. Centre well: see Fig. 1. Antigens: 1,4 = tumour, 2 = lung, 3 = heart, 5 = liver, 6 = spleen

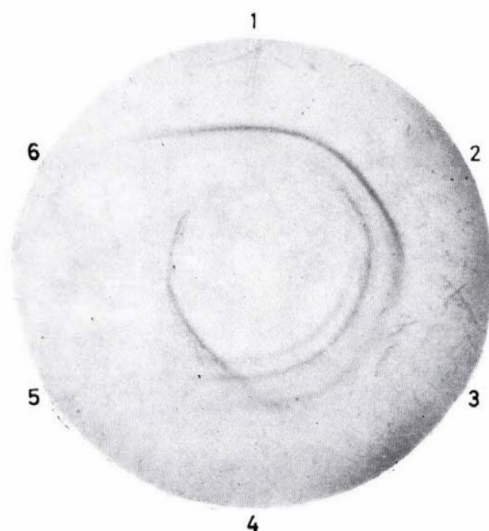


Fig. 5. Modified Ouchterlony plate. Centre well: antitumour serum prepared in tolerant rabbit. Antigens: 1,2 = tumour, 3,4 = liver, 5,6 = plasma

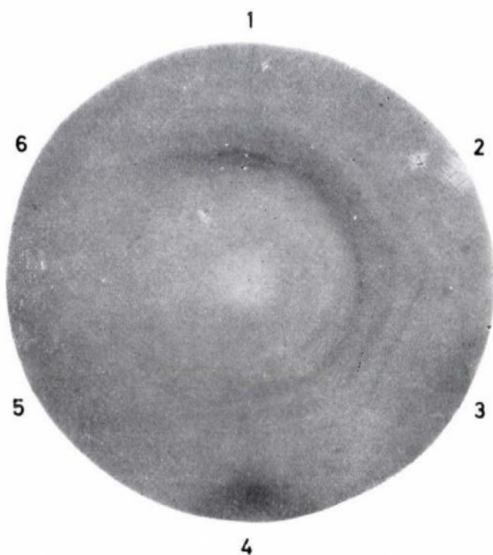


Fig. 6. Modified Ouchterlony plate. Centre well: tolerant rabbit anti-tumour serum absorbed by rat plasma and concentrated to half of the original volume. Antigens: 1,2 = tumour, 3,4 = liver, 5,6 = plasma

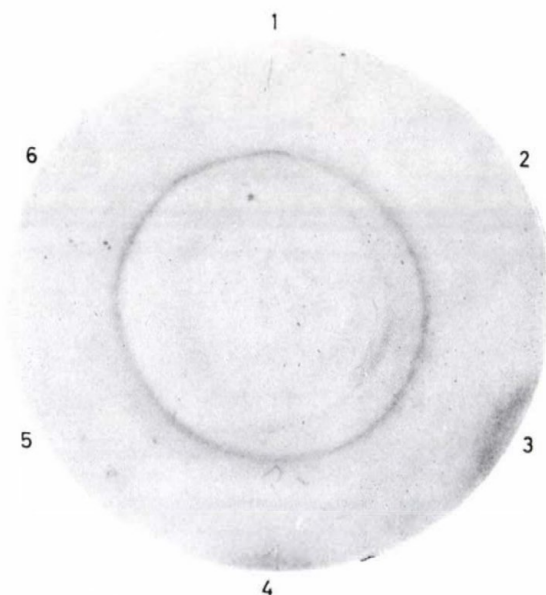


Fig. 7. Modified Ouchterlony plate. Centre well: see Fig. 6. Antigens: 1,2 = tumour, 3,4 = spleen, 5,6 = kidney

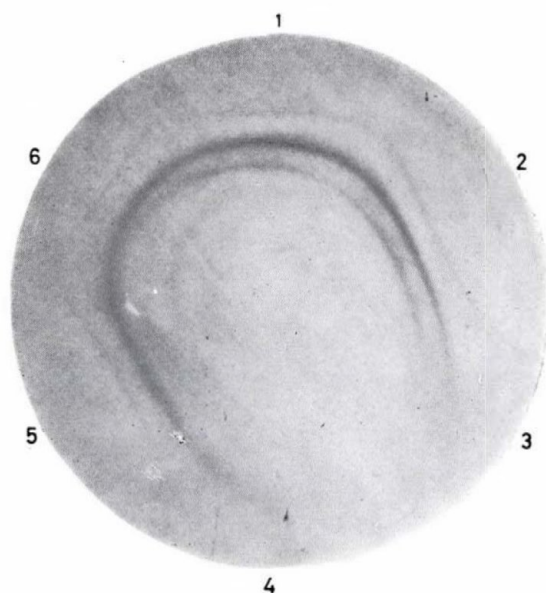


Fig. 8. Modified Ouchterlony plate. Centre well: see Fig. 6. Antigens: 1,2 = tumour, 3,4 = striated muscle, 5,6 = intestine

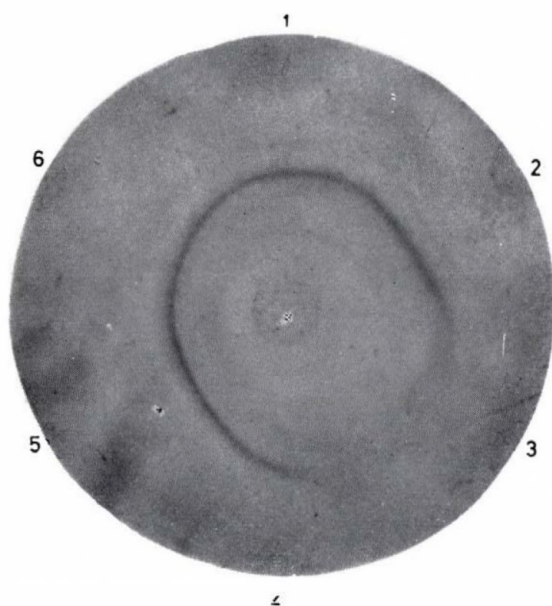


Fig. 9. Modified Ouchterlony plate. Centre well: see Fig. 6. Antigens: 1,2 = tumour, 3,4 = heart, 5,6 = lung

contained at least two antigens which were absent from the plasma. According to Fig. 2 in the tumour extract there were several plasma proteins not demonstrable in liver extract. Further experiments with the above anti-tumour serum were performed with BJÖRKLUND's inhibition technique. Three days prior to the addition of antiserum, rat plasma was pipetted into the centre well and let to diffuse into the agar gel. In such plates anti-tumour serum reacted only with one component of the rat plasma and the result given by cellular antigens could adequately be evaluated (Fig. 3). Inhibition experiments showed that the anti-tumour serum reacted with three components of the tumour extract. Each of these components was demonstrated in the spleen; two of them were present in the kidney and lung and one in the small intestine, striated muscle, heart and liver. It is interesting that the tumour antigen characterized by the strongest reaction gave a similarly strong reaction only in the spleen. Split into two constituents each exhibiting a considerably weaker reaction, the same component was demonstrated also in the lung (Figs. 3 and 4).

The anti-tumour serum prepared in rabbits tolerant to rat plasma reacted only with one component of rat plasma. Antibodies responsible for this reaction could be removed with small amounts of rat plasma. In subsequent experiments an anti-tumour serum absorbed in this manner and concentrated to half of its original volume was used. This serum reacted with four components of the tumour. The liver and spleen contained all these components. In the kidney and small intestine three, in the lung two of them were demonstrated. One component split into two parts was present in the heart. None of the components was revealed in striated muscle (Figs. 5 to 9).

Discussion

It is known from the investigations of SMITH and BRIDGES [15] that the higher the initial dose of the antigen and the longer the body remains in contact with it, the longer the duration of the unresponsive state. In the present experiments tolerance was prolonged for more than 8 months. This was shown by the fact that immunization with tumour antigen produced antibodies reacting only with one component of rat plasma.

The usefulness of immunological tolerance in studies on the antigenic structure of organs was first demonstrated by FELDMAN and YAFFE [9]. They showed that in rabbits rendered unresponsive to mouse heart extract, a subsequent immunization with mouse brain produced antibodies specific for brain antigens. In 1958, by use of the complement fixation test with anti-Rous sarcoma serum prepared in rats tolerant to normal muscle, ZILBER *et al.* [16] serologically differentiated the extracts of Rous sarcoma and normal muscle.

In tolerance experiments SVET-MOLDOVSKY and GENDON [17] demonstrated a serum antigen component present in rats with tumour but absent from normal rats. The experiments of LEVI *et al.* [18] and LEVI and SCHECHTMAN [19] are promising. They immunized rabbits unresponsive to mouse tissue with Ehrlich ascites tumour. The serum was not toxic in high doses and prolonged the life of mice affected with the corresponding tumour. An immune serum prepared with the same antigen in non-tolerant rabbits also prolonged survival when given in small doses, but in higher doses killed the mice immediately.

In the present experiments the antigenic structure of Benevolenskaya rat sarcoma has been examined. The immune serum prepared in rabbits tolerant to normal rat plasma reacted only with one component of the plasma and with four components of the tumour tissue. The four antigenic components revealed in the tumour were fully present in the spleen and the liver. When examined with BJÖRKLUND's inhibition technique, the anti-tumour serum prepared in non-tolerant rabbits gave an immune diffusion pattern different from that obtained with the anti-tumour serum prepared in tolerant rabbits. This finding may be explained by the fact that unresponsiveness had been induced with whole blood and erythrocytes and leukocytes are known to contain tissue antigens [20, 21]. On the other hand, the present experiments confirm the opinion of other authors [19, 22] that the unresponsive state is not merely a condition in which the production of antibodies against certain antigens is restricted, but a phenomenon which makes the detection of weakly immunogenic antigens easier and offers accordingly a chance to extend studies on the antigenic structure of tumours and organs.

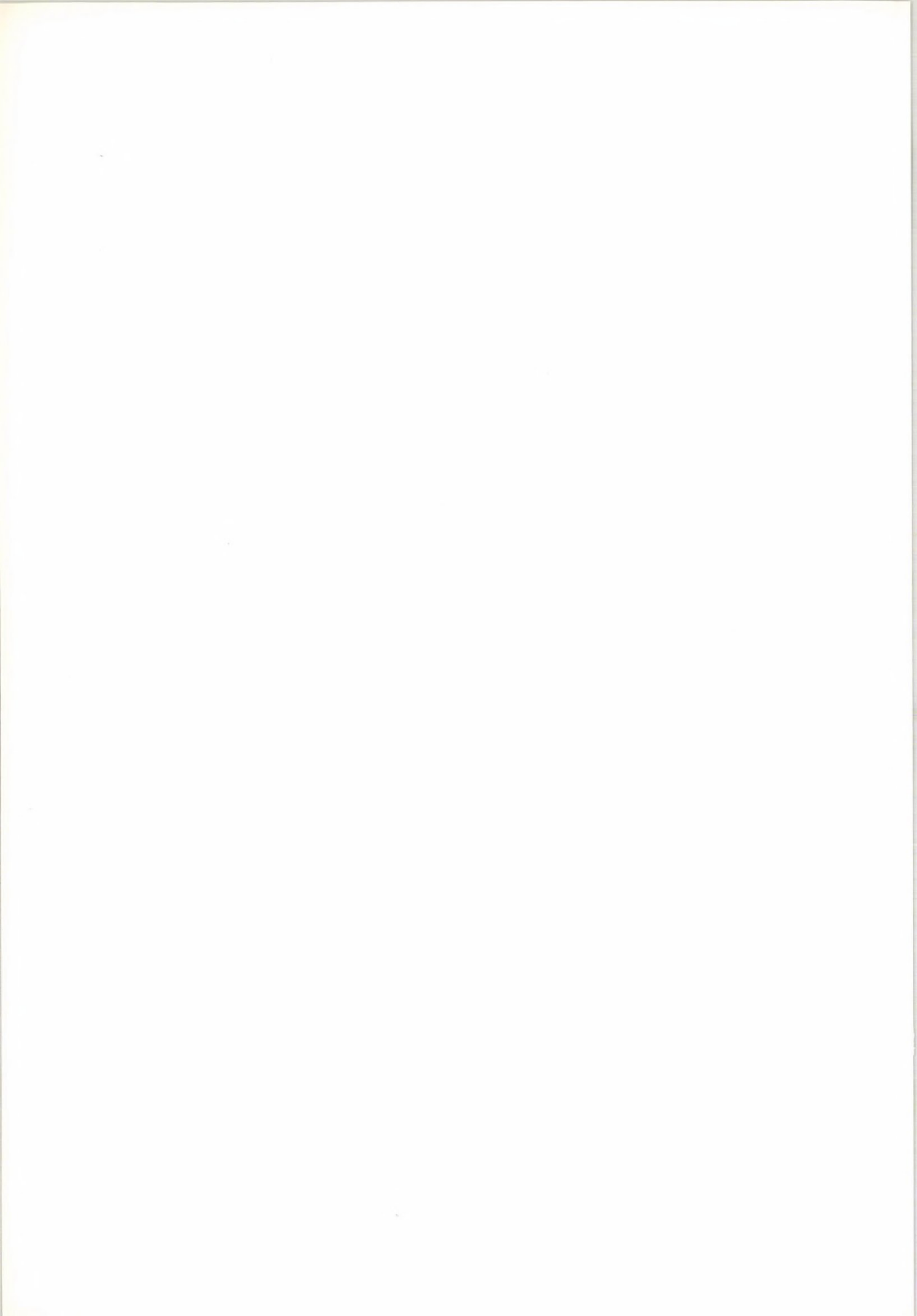
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THE EFFECT OF HEAT ON THE PHAGE SENSITIVITY OF STAPHYLOCOCCI

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(Received January 16, 1967)

Summary. Of 544 staphylococcal strains insensitive of phages of the standard basic and additional sets 46.3 per cent became typable after heat treatment. Lysogenic strains occurred among untreated cultures in 63.0, among heated cultures in 75.1 per cent. The host range of temperate phages obtained from heated cultures was 50.3 per cent wider than that of temperate phages released by untreated bacteria.

Phage typing of heated cultures and classification by the lytic spectrum of temperate phages have been found suitable for epidemiological purposes.

A working hypothesis advanced for the explanation of changes in phage sensitivity after heat treatment has been confirmed experimentally. It has been demonstrated on doubly infected untreated cultures that the heat-induced temperate phage recombines with the phage used for typing. The recombinant, having acquired a new host range, lyses the culture resistant to typing phages. The lysogenizing capacity and lytic properties of the recombinant phage have been examined.

A large number of *Staphylococcus aureus* strains are untypable by the usual phages. The incidence of untypable strains was found to amount to 20 per cent in neonatal pemphigus, by WILLIAMS *et al.* [1], 27 per cent in skin infections by WALLMARK [2], in hospitalized patients to 13 per cent by HOFSTAD and VOGELSANG [3] and to 11 per cent by ROSENDAL [4], in miscellaneous material to 25 per cent by WAHL and FOUACE [5] and in hospitalized patients to 33 per cent by FARRER and MCLEOD [6]. In our 8 year material untypable cultures occurred in 20 per cent on the average.

As the frequent occurrence of untypable staphylococci causes difficulties in epidemiological investigations, it is desirable to elaborate methods for the classification of these organisms. In the course of hospital outbreaks in 1959 and 1960, 4 new phages were isolated [7]; by the use of these the incidence of untypable staphylococci could be decreased to 5.9 per cent in 1960. As a further extension of the number of typing phages would have made typing too labourious, a more simple and practical method has been sought for.

MA and MANDLE [8] described that untypable staphylococcal strains became typable in 29 per cent after incubation at 45°C for 4 hours. Cultures remaining untypable after this treatment were rendered sensitive to typing phages in 26 per cent by an exposure to 55°C for 5 minutes.

ASHESHOV and JEVONS [9] showed that untypable staphylococcal strains involved in an outbreak became sensitive to type phage 187 when heated prior

to testing. They assumed that the heat had destroyed the cytoplasmic substance responsible for maintaining the prophage state.

On the basis of these observations we modified and improved the method of ASHESHOV and JEVONS [9] and undertook a systematic typing of all untypable staphylococci. In order to show the mechanism of the effect caused by heating and to classify untypable strains by another method, we extended the examinations to the lysogenic properties of the strains.

Materials and methods

Strains. A total of 544 strains untypable by basic and additional sets of phages were examined. The strains were isolated from patients and staff in maternity units and adults, wards, hospital air samples and food specimens. Lysogenization and recombination experiments were carried out with three of the above strains (167, 360 and 428).

Typing phages. *Basic set:* Group I: 29, 52, 52A, 79, 80; group II: 3A, 3B, 3C, 55, 71; group III: 6, 7, 42E, 47, 53, 54, 75, 77; group IV: 42D; miscellaneous: 81, 187. *Additional set:* 73, 42B, 47C, 47B, 52B, 69, 78.

Temperate phages: carried by strains 167, 360, 20, 46 and 158 were designated as (167'), (360'), (20'), (46') and (158'); temperate phages carried by the same strains after heating were named (167t'), (360t'), (20t'), (46t') and (158t'). In addition, recombinant phages 167t'/69 and 360t'/69 were used.

Heat treatment. Broth cultures were incubated at 56°C for 2 minutes. The plates were inoculated by flooding with the suspensions cooled rapidly after heating.

Tests for lysogenicity. Four hour broth cultures were centrifuged and the supernatant fluids were spotted on plates seeded with indicator cultures representing 28 standard propagating strains: 29, 52, 52A/79, 80, 3A, 3B, 3C, 55, 71, 6, 7, 42E, 47, 53, 54, 75/76, 77, 83A, 42D, 81, 187, 73, 42C, 42B/42C, 47B, 52B, 69, 78.

Detection of temperate phages released after heating was performed as follows. Broth cultures were heated at 56°C for 2 minutes, rapidly cooled, incubated at 37°C for 4 hours, and centrifuged, the supernatant was spotted on plates inoculated with the indicator strains. Quantitative determination of the number of free phage particles was performed after exposure to chloroform. Mitomycin induction was carried out at concentrations of 1 µg/ml.

Lysogenization was performed by spotting filtrates of lysogenizing temperate phages on plates seeded with broth cultures of the organisms. After incubation at 30°C overnight the secondary growth in the lytic zone was picked up and streaked onto agar plates. After three consecutive passages the culture was examined for lysogenicity and phage sensitivity.

Lysogenization was performed also in liquid medium as described by ROUNTREE [10]. Strain P. S. 73 served as the test culture and for the propagation of temperate phages.

Results and discussion

Typing of heated cultures. Of the 544 strains examined 252 (46.3 per cent) became typable after heat treatment. The phage group distribution of the strains is presented in Table I.

Epidemiological examinations indicated that the typing of heated cultures gave reliable results. For example, all 21 untypable staphylococcal strains isolated from 4 different maternity homes, turned out to belong to type 47/54 after heating.

Table I
Phage group distribution of heated strains

Phage group	No.	Per cent
I	36	6.6
II	10	1.8
III	106	19.5
IV	3	0.6
Miscellaneous	12	2.2
Mixed	51	9.4
Additional	34	6.2
Untypable	292	53.7
Total	544	100.0

Examination of standard staphylococcal strains used for phage propagation showed that, with the exception of strains 3A, 3C and 42D, heat treatment induced no change into another phage group.

Lysogenicity examinations. Of the 544 strains examined 342 (63.0 per cent) were lysogenic. After heat treatment, lysogenicity was demonstrated in 75.1 per cent of the cultures. In 206 strains (50.3 per cent) the lytic spectrum of the temperate phages released after heating was wider than that of the corresponding temperate phages released spontaneously.

Temperate phages obtained from unheated cultures were active in about 50 per cent against phage group III strains; the rest lysed strains belonging to

Table II
Lytic spectrum of temperate phages

Temperate phages	Lytic spectrum
(167')	47/73
(167t')	47/73/52B
(360')	83A/73
(360t')	47/83A/73
(20')	52
(20t')	52/47/54/75/73/52B
(46')	73/52B
(46t')	54/75/73/42C/52B
(158')	6/7/54/73/42C
(158t')	3A/3C/6/7/54/83A/73/42C

mixed and miscellaneous groups. The lytic spectrum of temperate phages released after heating exhibited a similar group distribution.

Table II presents the lytic spectrum of temperate phages released from 5 untypable strains spontaneously and on heating. The difference in the lytic spectra indicated the presence of two different prophages in untypable strains. This consideration has been proved in subsequent lysogenization experiments.

Table III
Quantitative examination of phage induction

Time of incubation	Treatment	Viable bacterial counts per ml	Free phage particle counts per ml
0 hr.	Untreated	6.7×10^7	3×10^2
2 hr.	Untreated	1×10^8	3×10^2
	Heated	1×10^7	4×10^3
	U. V. 1 min.	8×10^6	2×10^3
	Mitomycin	2×10^6	2×10^2

Quantitative examination of phages released spontaneously and after heating are demonstrated in Table III. Plaque counts for phages liberated after heating were 10 times higher than for phages obtained without heat treatment. Ultraviolet irradiation for 1 minute exerted an effect similar to heating. In contrast, mitomycin had no inducing effect. Experiments with 10 untypable strains gave the same results as those with the strain shown in Table III; in 3 strains heating induced a 100-fold increase in plaque count.

The inducing effect of heat on *Staph. aureus* was examined by CAVALLO [11]. He demonstrated that an exposure to 55°C for 15 seconds killed 90 to 100 per cent of the bacteria. The surviving cells disintegrated after 2 or 3 divisions and the number of free phage particles increased. A comparison of the lytic spectrum of temperate phages released spontaneously and on the effect of heating as well as the rise in phage titre indicated that heat induces prophages in untypable strains.

Mechanism of the change in phage sensitivity after heating. It has been revealed that the phages are adsorbed to treated as well as to untreated cells. In the latter case, however, no penetration or phage multiplication takes place. That is, untypable strains are not resistant, but immune to the examined phages.

A number of heated strains were lysed by type phage 69 of the additional set. There was an alteration in the host range of phage 69 multiplying in the heated culture: it became active against the corresponding unheated culture, but lost its ability to multiply on its original propagating strain. We attempted

to bring about a similar modification of the phage with untreated bacteria by means of double infection. Untreated, untypable strains 167 and 360 were infected with

(a) type phage 69 lysing heated strains 167 and 360;

(b) phages (167') and (360') released spontaneously from strain 167 and 360;

(c) temperate phages (167t') and (360t') released from heated strains 167 and 360;

(d) mixture of phage (167') and type phage 69; mixture of phage (360') and type phage 69.

(e) mixture of heat-induced phage (167t') and type phage 69; mixture of heat induced phage (360t') and type phage 69.

In experiments (a) to (d) no lysis was observed. In experiment (e) lysis occurred after simultaneous infection with two phages. In order to show whether phages derived in this manner are a result of complementation or recombination, a purified phage material was prepared by subculturing from the plaques three times. Host range properties indicated that in these experiments a new phage was obtained. It differed in host range from both phages used for double infection. This finding allowed the supposition that recombination had occurred.

The difference between recombinant and temperate phages released spontaneously and after heating was confirmed also by lysogenization and phage sensitivity experiments. While spontaneously released temperate phages of untypable strains produced no immunity to phages released by heat-induction, lysogenization with heat-induced phages established immunity against both kinds of temperate phage. In phage sensitivity the culture lysogenized with the recombinant phage differed from the culture lysogenized with temperate phages released spontaneously and after heat treatment

Table IVa

Phage sensitivity of strain P. S. 73 lysogenized with phages (167'), (167t') and 167t'/69

Strains	Phages				
	69	(167')	(167t')	167t'/69	73
73	+	+	+	+	+
73 (167')	—	—	+	—	+
73 (167t')	—	—	—	—	+
73 (167t'/69)	—	+	—	—	+
69	+	—	—	—	—

Key: + = lysis; — = no lysis.

Table IVb

Phage sensitivity of strain P. S. 73 lysogenized with phages (360'), (360t') and 360t'/69

Strains	Phages				
	69	(360')	(360t')	360t'/69	73
73	+	+	+	+	+
73 (360')	—	—	+	—	+
73 (360t')	—	—	—	—	+
73 (360t'/69)	—	+	—	—	+
69	+	—	—	—	—

Key: + = lysis; — = no lysis.

(Tables IVa and IVb). The difference in phage sensitivity of the same strain lysogenized with various phages indicates a difference in the lysogenizing phages.

The above results confirm that heat exerts a prophage-inducing effect. Model experiments also indicated that, as a result of the induction, the intracellular prophage entering the vegetative phase recombines with the typephage. Recombination results in the production of a phage with new lytic properties active against the formerly phage-insensitive strain.

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CHANGES IN THE FATTY ACID COMPOSITION OF STAPHYLOCOCCUS AUREUS UNDER VARIOUS CULTURAL CONDITIONS

By

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(Received January 19, 1967)

Summary. Fatty acid composition of *Staph. aureus* depends on environmental conditions. The amount of unsaturated fatty acids increases when the pH is decreased and oxygenation is increased. Of the total fatty acid content unsaturated fatty acids comprise 5.9 and 10.4 per cent in bacteria grown at pH 7.2 under static and shaken conditions, respectively. The corresponding percentages for cultures grown at pH 6 are 9.4 and 12.2.

Small variations in growth temperature do not significantly alter the amount of unsaturated fatty acids.

Composition of the medium considerably influences the saturated and unsaturated fatty acid content. In staphylococci cultured in the presence of 5 per cent human serum unsaturated fatty acids comprise 26.9 per cent of total fatty acids, in contrast to the 5 and 10.3 per cent values obtained in synthetic and vitamin medium, respectively. Staphylococci multiplying in the abdominal cavity of guinea pigs contain 36.2 per cent unsaturated fatty acids.

It has been concluded that the closer *in vivo* conditions are imitated by the cultivation method, the higher the degree of unsaturated fatty acid synthesis.

The lipid composition of bacteria depends on the conditions of culturing. The changes are indicated by alterations in fatty acid composition. In addition to pH and oxygenation, the ingredients of the medium are considerably influencing the lipid and fatty acid spectrum of bacteria. As the problem had only been studied *in vitro*, it seemed desirable to examine the effect of cultivation methods approaching or corresponding to natural conditions.

Materials and methods

Strains. The following coagulase, phosphatase, mannitol positive, gelatin-liquefying and haemolysin-producing *Staph. aureus* strains were examined: Nos. 53, 100 and 80/81.

Culturing *in vitro*. *Medium A:* hydrolysate of casein (Difco), 10 g; NaCl, 5 g; Na_2HPO_4 , 5 g; iron ammoniumsulphate, 0.03 g; MgSO_4 , 0.01 g; MnSO_4 , 0.01 g; distilled water to 1000 g. Before inoculation 5 g of sterile glucose were added. The pH was adjusted to 6.0, 7.2 and 8.0 with 0.1 N HCl and 0.1 N NaOH. The medium was distributed in 150 ml portions into 500 ml Erlenmeyer flasks.

Medium B contained the same ingredients as medium A, except that Tripkazin (Human, Budapest) was substituted for Difco Casiton, Vitamin B₁ and nicotinamide were added at concentrations 1 μg /100 ml and the pH was adjusted to 7.2.

Other media used were as follows. Medium A + 5 per cent human serum, pH 7.2; medium A + 10 per cent human plasma, pH 7.2. The cultures were incubated at 27, 37 and 41°C. In media A, B and A + 5 per cent human serum the bacteria were grown under shaking for 18 hours. The cells were then centrifuged at 3000 r. p. m. for 20 minutes, washed in saline, resuspended in 100 ml twice distilled acetone. The suspension was refrigerated at 4°C for 20 hours.

Culturing in vivo. Male guinea pigs of identical weight were injected intraperitoneally with 5 ml doses containing approximately 2×10^{10} cells of a 18 hour shaken culture of strains 53 and 80/81. The animals were bled 6 hours after injection. The abdominal exudate was drawn off, then the abdominal cavity was washed twice with 8 ml of pH 6 saline. The fractions were united and the suspension, which contained leukocytes and phagocytized bacteria in large numbers, was subjected to ultrasonic treatment for 3 minutes which, as shown by microscopic examination, completely disintegrated leukocytes. The bacteria were centrifuged and washed twice in distilled water, then the deposit was resuspended in acetone. In each experiment 4 guinea pigs were used.

Fatty acids were extracted and analysed gaschromatographically as described previously [10].

Results

1. Effect of pH and incubation temperature on the fatty acid composition of *Staph. aureus*. The fatty acid composition is changing with the culture's age. Alterations occurring as a result of environmental effects can therefore be compared only when all cultures are in the same phase of growth. Thus, before examining the fatty acid composition, the growth curve of *Staph. aureus* strains was determined at pH 5, 6, 7.2, 8 and 9.

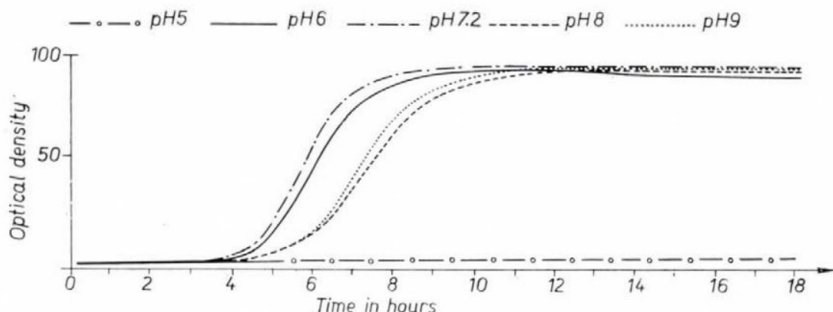


Fig. 1. Growth of *Staph. aureus* strain 53 at various pH values. Jouan biophotometer

Fig. 1 shows that *Staph. aureus* strain 53 failed to multiply in media adjusted to pH 5. At pH 6 and 7.2 the lag phase lasted for 4 hours, at pH 8 and 9 for 5 hours. Further stages of growth lasted for about the same time in all media. After 18 hours all cultures were in the late stationary phase. Similar results were obtained with *Staph. aureus* strain 100.

Table I shows the percentage distribution of fatty acids in strain 100 grown for 18 hours at 37°C in shaken medium "A" at pH 6, 7.2 and 8.

Cultures grown at different pH values synthesized the same fatty acids, but there was a difference in the proportion of these substances. In bacteria cultured at pH 6 arachic acid comprised 32 per cent of the total fatty acid content. With a shift of the medium to the alkaline side the amount of arachic acid increased: at pH 7.2 it was present in 39, at pH 8 in 48 per cent. In cultures

Table I*Effect of pH on the fatty acid composition of Staph. aureus strain 100*

Fatty acid	Abbreviation	pH		
		6	7.2	8
Arachic	C ₂₀	31.9	39.7	48.5
Stearic	C ₁₈	24.5	16.0	11.3
12-Methyltetradecanoic	ai C ₁₅	25.2	20.7	15.6
Palmitic	C ₁₆	5.0	7.3	9.1
Myristic	C ₁₄	3.3	8.1	8.4
Tetradecenoic	C ₁₄ : 1	6.6	5.8	4.6
Others (total)		4.5	2.4	2.5

grown at pH 8 the amount of stearic acid and anteiso-C₁₅ fatty acid decreased. Similar results were obtained for strain 53.

Table II presents the distribution of saturated and unsaturated fatty acids in strain 53 cultured in medium "A" for 18 hours under different conditions.

Table II*Distribution of fatty acids in Staph. aureus strain 53 grown under various conditions*

Cultural condition	Total fatty acids, per cent	
	Saturated	Unsaturated
pH 7.2, 37°C, static	94.1	5.9
pH 7.2, 37°C, shaken	89.6	10.4
pH 6.0, 37°C, static	90.6	9.4
pH 6.0, 37°C, shaken	87.8	12.2
pH 7.2, 37°C, human serum, shaken	73.1	26.9

As shown in Table II a shift to the acid side and the increase of oxygenization causes an alteration in favour of unsaturated fatty acids. The increase of unsaturated fatty acids at acid reaction is higher under static than under shaken conditions.

When the semisynthetic medium was supplemented with 5 per cent human serum, the amount of unsaturated fatty acids increased about 3-fold.

In serum broth conditions *in vivo* are approached better than in simple semisynthetic medium. In the former the synthesis of unsaturated and short-chain fatty acids increased while that of straight-chain C₂₀ arachic acid decreased.

Table III shows the saturated and unsaturated fatty acid content of *Staph. aureus* cultured under static conditions for 120 hours in synthetic medium with and without plasma.

Table III

Distribution of saturated and unsaturated fatty acids in Staph. aureus grown in the presence of 10 per cent plasma

Cultural condition	Total fatty acids, per cent	
	Saturated	Unsaturated
pH 7.2, 37°C, 120 hr., static	93.0	7.0
pH 7.2, 37°C, 120 hr., 10 per cent human plasma, static	82.5	17.5

It is seen that after 5 days culturing in synthetic medium unsaturated fatty acids constituted 7 per cent of the total fatty acid content. In bacteria grown in the presence of 10 per cent human plasma this value corresponded to 17.5 per cent.

Staph. aureus is not particularly sensitive to the temperature of cultivation. Our findings agreed with this observation; a 4°C increase of the temperature of growth caused no alteration in fatty acid composition. Staphylococci cultured at 37°C contained the same amount of lipid and fatty acid as those grown at 41°C, and they did not differ significantly in this respect from 27°C cultures.

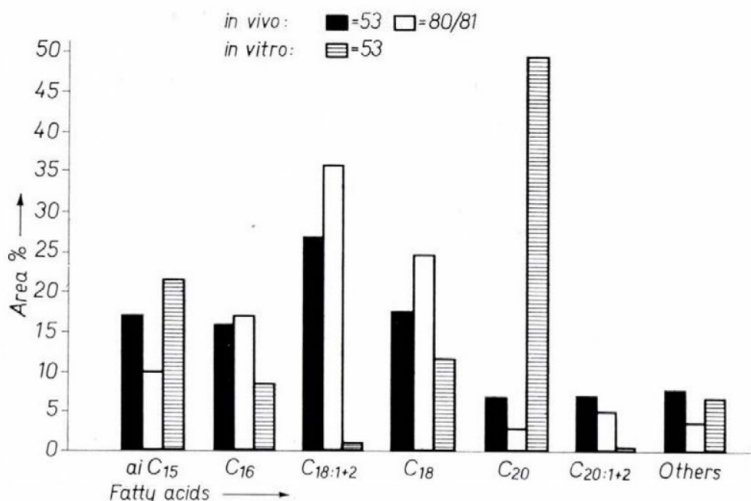


Fig. 2. Fatty acids in *Staph. aureus* cultured *in vivo* and *in vitro*

As in the fatty acid metabolism of staphylococci cultured under circumstances imitating conditions *in vivo* a considerable shift had occurred in favour of unsaturated fatty acid synthesis, it seemed desirable to investigate the fatty acid composition of strains multiplying *in vivo* (Fig. 2).

The main component in *Staph. aureus* strains 53 and 80/81 cultured *in vivo* was unsaturated C₁₈ fatty acid. Arachic acid, the main component of bacteria grown *in vitro*, was present in relatively small amounts (7 and 3 per cent of total fatty acids in strains 53 and 80/81, respectively).

Table IV shows the distribution of unsaturated fatty acids in *Staph. aureus* cultured under various conditions.

Table IV
Unsaturated fatty acid content of *Staph. aureus* grown under various conditions

Cultural condition	Unsaturated fatty acid content in per cents of total fatty acids
Synthetic medium	5.0
Synthetic medium + vitamins	10.3
Synthetic medium + 5 per cent human serum	26.9
Abdominal cavity of guinea pigs	36.2

Discussion

Alterations in the fatty acid composition of bacteria have been investigated by several authors. MARR and INGRAHAM [1] described that in *E. coli* grown at lower temperatures unsaturated fatty acids predominated, while in ageing cultures of the same bacterium cyclopropane fatty acids comprised the main components. The increase of cyclopropane fatty acids in ageing cultures was demonstrated by LAW, ZALKIN and KANESHIRO [2] and KNIVETT and CULLEN [3] in *E. coli*. LAW *et al.* [2] in *Agrobacter tumefaciens*, CROOM and MCNEILL [4] in *Lactobacillus arabinosus*, and KATES, ADAMS and MARTIN [5] in *Serratia marcescens*. CROOM *et al.* [6] showed that changes in the ingredients of the medium cause alteration in the fatty acid composition of the biotin-requiring bacteria *L. plantarum* and *E. coli*. KNIVETT and CULLEN [7] demonstrated that cyclopropane fatty acids increase in *E. coli* grown at acid pH. Cultural conditions are influencing not only the fatty acid spectrum but also the lipid and phospholipid content. For example, one of the main phospholipids in staphylococci grown at pH 7 is phosphatidylglycerol, but in cultures grown at pH 5 the lysine ester of this compound predominates [8]. OP DEN KAMP *et al.* [9] showed that the phospholipid composition of *B. megaterium* is considerably influenced by the pH and glucose content of the medium.

Alterations in cultural conditions are influencing the fatty acid synthesis in, and consequently the fatty acid composition of, staphylococci. In 18 hour shaken cultures grown in medium "A" unsaturated fatty acids comprised only 5 per cent of the total fatty acid content. If the medium was supplemented with vitamin B₁ and nicotinamide, the proportion of unsaturated fatty acids increased to 10.3 per cent. When the bacteria were grown in the presence of 5 per cent human serum, they contained these substances in 26.9 per cent. Staphylococci multiplying *in vivo* showed the highest unsaturated fatty acid content: 36.2 per cent.

It may, therefore be concluded that the more closely the cultivation method imitates conditions *in vivo*, the higher the degree of unsaturated fatty acid synthesis, that is, higher amounts of unsaturated fatty acids are built into the lipids and phospholipids of the organism.

The effect of changes in fatty acid and phosphatide synthesis on the properties of bacteria is not known. However, it is evident from the investigations of RIECH [11], FLEISCHER [12], OSTROVSKY [13], HENDLER [14], GABY [15], HUNTER [16] and others, that lipids play an important role in the basic biological functions and structure of cells.

Our results indicate that the changes in fatty acid and phosphatide synthesis are probably the first reactions of the cell to environmental effects. The alterations in the structure of membrane lipids influences the physico-chemical properties of the membrane on the one hand, and the activity of enzymes present in the membrane on the other. These changes, accordingly, exert an influence on the cell's entire biological activity.

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PROPERTIES OF *ESCHERICHIA COLI* SEROGROUP O18 STRAINS ISOLATED FROM AN OUTBREAK OF ENTERITIS AMONG NEWBORN INFANTS

By

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(Received February 6, 1967)

Summary. *E. coli* strains associated with an outbreak of infantile enteritis have been examined. The causative agent was characterized by antigenic formula O18a, 18c:K77(B21):H7. Epidemic occurrence of this serotype has not so far been described in Hungary.

EWING *et al.* [1] described that some *E. coli* strains isolated from enteritis were serologically related to KAUFFMANN'S *E. coli* strain F 10018. The strains differed from the latter in one O antigen component and in B and H antigens. Their antigenic structure was described as O18a, 18c:B21:H7, while strain F 10018 was characterized by formula O18a, 18b:B20:H14.

Some of the strains examined by EWING *et al.* had been isolated by DULANEY and MICHELSON [2] in the winter of 1933—34 from infants treated with enteritis in a Tennessee hospital. As the cultures showed variable lactose fermentation, DULANEY and MICHELSON described them as *B. coli mutabile*. The organism caused enteritis in 27 babies and this was fatal in nearly half of the cases. The causative agent was isolated from the faeces of 18 out of the 27 infants. EWING *et al.* [1] compared these strains with O18 cultures isolated from other newborn and young infants and showed that the cultures were antigenically identical with the strains of DULANEY and MICHELSON, but unlike those, had caused mild enteritis lasting only for a few days.

Our material consisted of faecal samples taken from new-born babies delivered between January and April, 1964, in the maternity unit of the Vác hospital. A total of 98 babies were involved in the outbreak. About 3 to 4 days after birth they excreted fluid, yellowish, non-fetid faeces several times a day. The temperature of the infants was 0.2 to 0.4°C above normal. No severe symptoms were observed.

Material and methods

A total of 74 faecal samples taken from 40 infants with enteritis and from 14 members of the nursing staff were examined. The causative agent was isolated on eosin methylene blue agar.

Biochemical reactions were performed as described in reference [3].

Antibiotic sensitivity was tested by use of Biotest paper discs (Institute for Sero-bacteriological Production and Research Human, Budapest).

Preparation and absorption of immune sera and determination of O, K and H antigens were carried out as described by KAUFFMANN [4], EWING *et al.* [5] and EDWARDS and EWING [6], except that OK sera were prepared with alcohol-treated, freeze-dried antigens.

Antigenic analysis was performed with type strains 3219/54, F 10 018/41 and U 5/41 received from the State Serum Institute, Copenhagen.

Results

The causative agent was isolated from 24 out of 40 infants. The organism grew almost in pure culture from all samples. As repeated examinations yielded further positive results, a total of 37 strains were isolated.

Table I

Biochemical reactions of strains F 10 018/41, 3219/54 and 12 058

	F 10 018/41	3 219/54	12 058
Glucose	++	++	++
Mannitol	++	++	++
Lactose	++	++ ⁹	++ ⁴⁻⁹
Maltose	++	++	++
Rhamnose	+	+	+
Arabinose	+	+	+
Sorbitol	+	+	+
Salicin	—	+ ³	+ ⁵
Dulcitol	—	—	—
Adonitol	—	—	—
Inositol	—	—	—
Raffinose	—	—	—
Sucrose	—	++	++
Urea	—	—	—
Methyl red	+	+	+
Voges-Proskauer	—	—	—
Indole	+	+	+
Xylose	+	+	+
Malonate	—	—	—
Ammonium citrate	—	—	—
H ₂ S	—	—	—
Tryptophan deaminase	—	—	—
Gelatin	—	—	—
Motility	+	+	+

++ = acid and gas in 1 day; ++⁹ = acid and gas in 9 days; + = acid production in 1 day or positive reaction; +⁵ = acid production in 5 days; — = negative reaction.

The organism produced colourless (lactose negative) colonies on eosin methylene blue agar. After several days red papillae appeared on some of the colonies. This phenomenon was described by EWING *et al.* as characteristic of bacteria belonging to *E. coli* serogroup O18.

The biochemical properties of one of our strains is compared with type cultures 3219/54 and F 10 018/41 in Table I.

The strains produced acid and gas from glucose, mannitol, arabinose and sorbitol in 24 hours at 37°C. Inositol, raffinose and adonitol were not fermented within 30 days. Indole and methyl red reactions were positive. The cultures failed to produce urease and hydrogen sulphide; they did not utilize citrate and were Voges-Proskauer negative. All cultures were motile in semisolid agar.

All strains gave positive slide agglutination in serum O18:B21. The titre of tube agglutination with boiled cultures was 1 : 25,600. Cross agglutination tests with boiled (1 hour at 100°C) suspensions of strain 12 058 (isolated from the outbreak) and type strains F 10 018/41 (O18a, 18b:B20:H14) and 3219/54 (O18a, 18c:B21:H7) in the corresponding OB sera indicated a close antigenic relationship. Cross absorption performed with bacteria heated at 100°C for 2 1/2 hours revealed that the O antigen of our strain was identical with that of strain 3219/54. The results are shown in Table II.

Table II

Analysis of the O antigen of strain 12 058

Antigens 1 hr. 100°	OK sera								
	12 058			3219/54			F 10 018/41		
	Unab- sorbed	Ab- sorbed by 3219/54	Absorbed by F 10 018/41	Unab- sorbed	Ab- sorbed by 12 058	Absorbed by F 10 018/41	Unab- sorbed	Ab- sorbed by 12 058	Absorbed by 3219/54
12 058	6400	0	1280	25,600	0	2560	3200	0	0
3 219/54	6400	0	1280	25,600	0	2560	3200	0	0
F 10 018/41	3200	0	0	25,600	0	0	6400	1280	640

F 10 018/41 = O18a, 18b

3 219/54 = O18a, 18c

The living suspension of the examined strain was inagglutinable in pure O serum prepared with strain 3219/54 boiled for 2 1/2 hours. This observation indicated that the strain contained K antigen. When OB sera 3219/54, F 10 018/41 and 12 058 were absorbed by the homologous boiled cultures, they failed to agglutinate the corresponding living cultures. As the agglutinin-binding capacity was not destroyed by heating, the cultures contained B antigens.

According to cross absorption experiments the B antigen of our strain was identical with that of strain 3219/54 but different from that of strain F 10 018/41 (Tables III and IV).

Table III

Analysis of the B antigen of strain 12 058

Absorption of OB sera by cultures heated at 100°C for 2 1/2 hours

Antigens (living)	OB sera			
	3219/54	12 058	12 058	F 10 018/41
	absorbed by			
	12 058	3219/54	F 10 018/41	12 058
12 058	0	0	160	0
3 219/54	0	0	320	0
F 10 018/41	0	0	0	160

F 10 018/41 = O18a, 18b:B20

3 219/54 = O18a, 18c:B21

Table IV

Examination of the B antigen of strain 12 058 in O serum

Antigens	Unabsorbed O serum 3219/54
12 058 living	0
12 058 2 1/2 hr. 100°C	2560
3 219/54 living	0
3 219/54 2 1/2 hr. 100°C	2560

In order to determine the H antigen, the cultures were passaged through semisolid agar. Cross agglutination indicated that the H antigen of our strain was identical with, or related to, the H antigen of strain 3219/54. Further H antigen analyses were carried out with type strain U 5/41 containing H antigen 7. Cross absorption experiments demonstrated that our strain and strain U 5/41 contained identical H antigens (Table V). Immobilization experiments with motile cultures in H sera also confirmed the above findings (Table VI).

The antigenic structure of the organism examined in the present study is, therefore, O18a, 18c:K77(B21):H7.

All strains isolated from the outbreak were sensitive to streptomycin, neomycin and polymyxin B, moderately sensitive to erythromycin, and resistant to penicillin, chloramphenicol, tetracycline and sulphonamides.

None of the strains tested produced keratoconjunctivitis in guinea pigs [7].

Table V*Analysis of the H antigen of strain 12 058*

H antigens	Pure H sera*			
	12 058		U 5/41	
	Unabsorbed	Absorbed by U 5/41	Unabsorbed	Absorbed by 12 058
12 058	6400	0	25,600	0
U 5/41	3200	0	25,600	0
F 10 018/41	0	0	0	0

F 10 018/41 = O18a, 18b:B20:H14

U 5/41 = O1:L1:H7

* H serum absorbed by the homologous boiled culture.

Table VI*Examination of motility*

Strains	U tube		
	With H serum 3 219/54	With H serum 12 058	Without serum
12 058	—	—	+ ¹
3 219/54	—	—	+ ¹
F 10 018/41	+ ¹	+ ¹	+ ¹
U 5/41	—	—	+ ¹

3 219/54 = O18a, 18c:B21:H7

F 10 018/41 = O18a, 18b:B20:H14

+¹ = migration through U tube in 1 day

— = no migration within 14 days.

Discussion

E. coli strains isolated in the present study from an outbreak of mild enteritis among newborn infants were identified as belonging to serotype O18a, 18c:K77(B21):H7. The pathogenic role of the organism was confirmed by the fact that it grew almost in pure cultures and no other pathogenic bacterium was present in the faecal samples.

EWING *et al.* [1] described the same serotype as the causative agent of mild cases of sporadic enteritis. The organism was also associated with a severe outbreak of enteric infection [2].

No outbreak due to *E. coli* serogroup O18 strains has so far been described in Hungary. It is obvious to assume that some sporadic cases of enteritis in this country might have been caused by *E. coli* O18. These, however, as the corresponding sera are not routinely used, have remained undetected.

Acknowledgement. The authors are indebted to DR. A. PETRILLA and DR. I. PERÉNYI for epidemiological and clinical data.

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INCIDENCE OF CRYPTOCOCCUS SPECIES IN URBAN AIR

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Summary. During a 18 month period a total of 329 white yeast-like organisms were isolated from urban air. Of the isolates 47.1 per cent (155 strains) were starch-producing organisms.

Exact identification was performed with 100 starch-producing isolates. All of them belonged to the genus *Cryptococcus*. As to species distribution, *C. albidus* (SAITO) SKINNER, 74 strains; *C. diffluens* (ZACH) LODDER et KREGER VAN RIJ, 17; *C. laurentii* (KUFFERATH) SKINNER, 9; while no *C. neoformans* (Sanfelice) VUILLEMIN were isolated. All species showed an even monthly and seasonal distribution.

In view of the possible air-borne contamination of the skin and other accessible parts of the body with these organisms, their presence in materials taken from suspected mycotic conditions should be evaluated with caution.

In 1966, an account was given of the incidence of *Rhodotorula* species in urban air [19]. The present paper deals with *Cryptococcus* species frequently isolated from the same source.

As it has been pointed out, air as a habitat of facultative pathogenic fungi, may be a source of human and animal infections and should therefore be considered in epidemiological investigations. On the other hand, in view of their common occurrence in air, the presence of saprophytic and facultative pathogenic fungi in the clinical materials of patients with suspected mycosis should be evaluated with proper criticism.

In 1923 SAITO demonstrated the presence in air of *Torula gelatinosa* SAITO, *T. albida* SAITO and *T. luteola* SAITO [13]. He showed that in every month of the year *T. gelatinosa* and *T. albida* occurred most frequently in air. According to the nomenclature adopted at present, *T. gelatinosa* and *T. albida* are synonyms of *Cryptococcus albidus* (SAITO) SKINNER, while *T. aurea* corresponds to *C. laurentii* (KUFFERATH) SKINNER and *T. luteola* to *C. luteolus* (SAITO) SKINNER [5]. The occurrence of *Cryptococcus* species in air has rarely been mentioned in later literature.

Air may become contaminated with *Cryptococcus* by means of dust particles originating from soil, of which *C. albidus* is a common inhabitant. *C. diffluens* and *C. laurentii* are less frequently encountered in soil [6, 9]. The faeces of birds, which contain *Cryptococci* in large numbers, may also be a source of air contamination [4, 17].

Materials and methods

Each day between April, 1965, and November, 1966, with the exception of the coldest months December and January, two air samples were taken, one in the morning, the other at noon. Sampling was performed by exposing molasses agar plates for 15 minutes on the top of a 20 metre high building situated at a busy traffic centre in Budapest (Nagyvárad square).*

The plates were incubated in a dark place for 2 to 4 days, then white colonies of yeast-like organisms were transferred to molasses agar slants. Starch-producing activity of the pure cultures was examined by the MAGER-ASCHNER method [5]. Starch-producing strains were identified as described by LODDER and KREGER VAN RIJ [5] except that utilization of ethanol as a sole source of carbon was determined by turbidimetric measurement [19].

Results

During the 18 month period 329 white yeast-like organisms were isolated; 155 (47.1 per cent) among them produced starch. The monthly distribution of starch positive strains was even, no seasonal variation was observed.

Of the 155 starch-producing isolates 100 strains showing strong reaction with LUGOL's solution (deep blue to black colour) were identified. These cultures, on the basis of starch production and failure to decompose sugars fermentatively, were classified into the genus *Cryptococcus*. The species distribution was as follows. *C. albidus* (SAITO) SKINNER, 74; *C. diffluens* (ZACH) LODDER et KREGER VAN RIJ, 17; *C. laurentii* (KUFFERATH) SKINNER, 9. Neither *C. neoformans* nor *C. luteolus* was isolated from air during the 18 months. All species showed an even monthly and seasonal distribution.

In physiological properties and cell size all 74 *C. albidus* strains were identical. On the basis of colonial morphology two groups were distinguished 48 strains produced highly mucoid colonies showing occasionally sectorial structure while 26 strains grew in non-mucoid colonies characterized frequently by a granular structure.

Discussion

The members of the genus *Cryptococcus* are frequently mentioned in the literature dealing with fungi present in pathological conditions or in the normal flora. Although *C. neoformans* is generally accepted as the causative agent of cryptococcosis [3], a case associated with another *Cryptococcus* species has been described in Hungary [2]. In our experience materials from suspected mycoses supplied several *Cryptococcus* species [18, 20]. The presence of *C. albidus*, *C. diffluens* and *C. laurentii* was described on skin, in respiratory and urogenital organs, in bile and even in carious teeth [1, 7, 8, 11, 14, 15, 12, 10, 16].

Our studies indicate that certain *Cryptococcus* species (*C. albidus*, *C. diffluens*, *C. laurentii*) are common in urban air. These organisms may therefore

* The samples were taken by the Hygienic Microbiological Laboratory of our institute.

Table I

Morphological and physiological properties of Cryptococcus species isolated from urban air

Species	No. of isolates	Sugar fermentation	Sugar assimilation								NO ₃ assimilation	Starch production	Ethanol assimilation	Arbutin decomposition	Colonial structure and cell size, malt agar culture, μ
			D	G	S	M	L	R	Ce	Me					
<i>C. laurentii</i>	9	—	+	+	+	+	+	+	+	d	—	+	—	+	Non-mucoid, waxy, ivory-white; 2.6—7.8 $\times$ 3.2—13.0 2.6—7.8
<i>C. diffluens</i>	17	—	+	+	+	+	—	+	+	—	+	+	d	+	Slightly mucoid, light brown; 3.2—5.2 $\times$ 3.9—7.8 3.9—7.8
<i>C. albidus</i>	26	—	+	+	+	+	+	+	+	—	+	+	d	+	Nonmucoid, waxy, ivory-white, granular; 2.6—7.8 $\times$ 3.9—15.6 2.6—10.4
<i>C. albidus</i>	48	—	+	+	+	+	+	+	+	—	+	+	d	+	Mucoid, ivory-white, sectorial; 2.6—7.8 $\times$ 3.9—15.6 2.6—10.4

Abbreviations: D = glucose, G = galactose, S = sucrose, M = maltose, L = lactose, R = raffinose, Me = melibiose, Ce = cellobiose, d = varying

easily contaminate the skin and other accessible parts of the body. Accordingly, their presence in samples taken from such sites is probably due to contamination. The pathological role of these *Cryptococcus* species may be assumed only if the organism can be cultured from the patient on several subsequent occasions or if an increased immune or allergic reaction to the organism can be demonstrated.

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SUSCEPTIBILITY OF THE DOMESTIC SWINE TO INFLUENZA B VIRUS

By

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(Received March 2, 1967)

Summary. Attempts were made to infect two young piglets intranasally with a freshly isolated strain of influenza B virus. One of the piglets developed fever and subsequently haemagglutination-inhibiting (HI) and neutralizing antibodies to the homologous strain. A contact pig, too, developed fever and homologous HI antibodies, but no neutralizing antibodies.

From eight farms where several months earlier pigs had been affected by an influenza-like disease during, or subsequent to, a human epidemic, 924 serum samples were collected at random. In four farms 8 to 24 per cent of the pigs had antihaemagglutinins to a freshly isolated influenza B strain. In one case a HI titre of 1 : 160 was associated with a still higher neutralization titre.

ELKELES [1] was the first to infect baby piglets with human influenza virus (WS strain), soon after the first isolation of influenza virus from human subjects. Subsequently, SHOPE and FRANCIS [2] succeeded in evoking a disease resembling mild swine influenza in pigs 6 to 14 weeks of age and recovering the human A strains from atelectatic areas of the lungs. Soon thereafter SHOPE [3] detected neutralizing antibodies to human influenza A virus in serum samples of pigs having no antibodies to swine influenza virus. In Hungary, HEGYELI and HOFFMANN [4] observed an epizootic in a swine herd with clinical manifestations resembling those of swine influenza during a widespread influenza epidemic. The diseased pigs developed neutralizing antibodies to the influenza A virus strain that was then prevailing in the human population but not to swine influenza virus [5].

PATOČKA *et al.* [6] infected pigs with the A-2 strain Singapore 1/57 as evidenced by clinical symptoms, pathological findings, and re-isolation of virus even from the third swine passage. Since then further observations have supported the susceptibility of pigs to human influenza A-2 virus. It has, however, remained unclear whether the domestic pig was susceptible to influenza B virus.

In November and December, 1965, and January, 1966, small influenza B outbreaks occurred in Hungary. In certain areas the epidemic was accompanied by outbreaks of a mild acute respiratory disease among pigs. The disease was characterized by cough, serous nasal discharge and conjunctivitis. The simultaneity of the human and animal outbreaks raised the possibility of a common aetiology.

This paper deals with serological tests carried out in blood samples collected at random in eight swine herds after outbreak and with attempts to induce influenza B infection in young piglets.

Materials and methods

Animal experiments. Four to six weeks old piglets from the breeding of a state farm were used. Two piglets (signed A and B) were inoculated intranasally with 1.0 ml of a pooled allantoic fluid harvested from an early amniotic passage of influenza B Budapest 10/65, a strain isolated in November, 1965. A third piglet (C) was left uninfected to serve as contact control. Further two piglets (D and E), also uninoculated, were kept in the same room, separated from the others by iron bars. Blood samples were taken from the cranial vena cava.

Swine herds. Some data of the eight swine herds from which random blood samples were collected are given in Table III.

Haemagglutination-inhibition (HI) tests. Serum samples were heated to 56°C for 30 minutes; 0.1 ml of each inactivated sample was mixed with an equal volume of 0.02 M KIO₄ and incubated for 3 hours at 37°C; then two drops (0.05 ml) of glycerol were added. The test was performed with the Microtitrator technique [7, 8]. The influenza strains swine A-1 Shope-15, A-O PR8, A-2 Singapore 1/57 IR, and B Budapest 10/65, were used.

Neutralization tests. Serial dilutions of inactivated sera were mixed with equal volumes of influenza B Budapest 10/65 virus, which contained 200 ID₅₀ per 0.1 ml as titrated in the test system. The mixtures were incubated at 37°C for one hour, then inoculated into systems consisting of chorioallantoic membrane fragments suspended in a buffered saline containing glucose and egg white. The inoculum was 0.1 ml. The inoculated tissue fragments were rolled at 37°C in HORVÁTH's drum [9] for 72 hours, whereafter the supernatants were tested for haemagglutination with chicken red cells. The highest serum dilution (calculated for the serum-virus mixture) which prevented virus from attaining a haemagglutinating concentration in the fluid phase was considered the neutralization titre. Neutralization tests *in ovo* were carried out as usual.

Results

Experimental infection. Three days after intranasal inoculation with influenza B virus the rectal temperature of piglets A and B rose by about 2°C to a level about 1°C higher than the simultaneous rectal temperature of the contact control (piglet C) (Table I). The piglets showed no further symptoms or signs.

Eight days after the inoculation of piglets A and B the rectal temperature of the uninoculated piglet E rose to 39.8°C, the piglet had no appetite and

Table I

Rectal temperature of two four-week-old piglets inoculated intranasally with strain influenza B Budapest 10/65, and of a control piglet

Piglet	Date of intranasal inoculation	Temperature									
		5/4	6/4	7/4	8/4	9/4	10/4	11/4	12/4	13/4	14/4
A	5/4/66	37.5	37.3	39.2	39.5	39.1			39.0	38.6	38.5
B	5/4/66	38.0	38.4	39.1	39.7	38.9			38.9	38.9	38.6
C	—	37.9	38.2	38.3	38.6	38.8			39.3	38.9	38.3

lagged in growth. Its temperature was elevated for four days. Piglet D, kept together with piglet E, remained free of fever and symptoms.

The HI and neutralization titres of the piglets' sera are shown in Table II.

The pre-inoculation samples were free of HI and neutralizing antibodies, whereas 20 days after intranasal inoculation of piglets A and B, piglet B had antihaemagglutinin (1:15) to influenza B virus. The others, including the inoculated piglet A, remained negative. Piglet A died on the same day because of a technical error. Ten days later piglet B still retained its HI titre and showed a considerable neutralization titre. At the same time piglet E, too, had antihaemagglutinin, but its neutralization titre (1:8) was too low to be appreciated.

Table II

Immune response of two piglets (A and B) to intranasal inoculation with strain influenza B Budapest 10/65 and of three piglets (C, D and E) exposed to contact infection

Piglet	Date	Route	Homologous titres on							
	of inoculation		5/4/66 HI N*	25/4/66 HI N*	5/5/66 HI N*	9/6/66 HI N*	8/7/66 HI N*	24/8/66 HI N+		
A	5/4/66	nasal	— NT	—	died, technical error					
B	5/4/66 9/6/66	nasal+intra- muscular	— —	15	15 32	— 8	320 512	160 256		
C	9/6/66	intra- muscular	— NT	—	— NT	— NT	10 NT	5 8		
D	9/6/66	intra- muscular	NT NT	—	— NT	— NT	30 NT	15 NT		
E**	9/6/66	intra- muscular	NT NT	—	15 8	— NT	240 16	80 8		

* Neutralization test was carried out in chorio-allantoic membrane fragments.

+ Neutralization test in ovo, NT = not tested.

** 13/4/66—17/4/66: fever (39.8°), anorexia.

After further five weeks the HI titres, and the low neutralization titre, of piglet E had disappeared, but piglet B still had low neutralizing antibodies (1:8).

An attempt has been made to verify the antibody response by giving to piglets B and E an intramuscular booster dose of 1.0 ml per animal of the allantoic-fluid virus Budapest 10/65. Pigs C and D were given the same dose.

Piglet B gave an intensive response as proved by both tests. In the second month after reinoculation the high antibody level showed a slow decline. Piglet E gave an antihaemagglutinin response similar to that given by B. How-

Table III

Serological survey on 924 pigs in 8 herds affected by influenza-like outbreaks in the period from November, 1965, to January, 1966

Farm	Data of taking blood	No. of pigs tested				Pigs with HI titres $\geq 1:8$								Remarks
		< 6 months	6—11 months	≥ 1 year	Total	Swine A-1 S 15/31		A-2 Sing 1/57		A-0 PR 8		B Bp 10/65		
						No.	%	No.	%	No.	%	No.	%	
		of age												
I	11/5/66	—	—	100	100	3	3.0	—	—	4	4.0	11	11.0	Sows, 2—8 year old
II	11/5/66	—	—	30	30	—	—	—	—	—	—	—	—	Sows, 1—4 year old
III	28/5/66	—	—	219	219	8	3.4	1	0.4	2	0.9	19	8.6	
IV	22/12/65	—	45	—	45	—	—	—	—	—	—	—	—	Porkers
V	12/5/66	—	60	40	100	—	—	—	—	2	2.0	24	24.0	Young breeding sows, porkers
VI	28/3/66	—	130	—	130	18	13.8	—	—	—	—	—	—	Young breeding sows
VII	10/5/66	67	33	—	100	—	—	—	—	3	3.0	12	12.0	Porkers
VIII	13/5/66	125 ^o	75 ^{oo}	—	200	—	—	—	—	—	—	—	—	^o 2—4 months ^{oo} 6—8 of age
Total		192	343	389	924									

ever, its neutralization titre remained at a relatively low level. Two months after the intramuscular inoculation the neutralization titre of piglets E and C was at an equally low level while piglet B still had a high titre.

Serological survey. Serum samples were collected at random from 924 pigs in eight swine herds. The pigs ranged in age from three and a half months to 8 years. In three farms none of the donor swine had antibodies to any of the four test strains (Table III).

In further four farms 8 to 24 per cent of the animals had detectable antihaemagglutinins to influenza B virus Budapest 10/65. From the same farms 2.1 per cent of the serum samples inhibited PR8 to some extent. In two of these farms and in farm VI several pigs with antihaemagglutinins to influenza A swine-1 were found. A single serum inhibited the human A-2 virus.

The HI titres to influenza B ranged from 1 : 10 to 1 : 40 except for one serum sample which had a titre of 1 : 160.

The neutralization test was carried out, using chorioallantoic membrane fragments, in 15 HI-positive and 4-negative sera. The results, compared to the HI titres, are shown in Table IV.

Table IV
Comparison of HI and neutralization titres in 19 pigs

		Neutralization titre								
		< 8	8	16	32	64	128	256	512	Total
HI titre	160								1	1
	80									—
	40	1	2	4	1	2				10
	20		2			2				4
	10									—
	<10	2	2							4
Total		3	6	4	1	4			1	19

It is clear that the sera having no detectable antihaemagglutinins had no or hardly any neutralizing activity. The HI-positive sera, on the other hand, were all but one positive in the neutralization test, four sera at the dilution of 1 : 8, 10 sera at 1 : 16—1 : 64 and the serum that had the highest (1 : 160) HI titre was positive up to 1 : 512.

Thus, some correlation between the HI and neutralization titres was obvious.

Discussion

The intranasal inoculation with influenza B virus of piglet B was followed by a considerable primary neutralizing and HI antibody response and the intramuscular booster dose of virus evoked an antibody response.

The response by piglet E, exposed to contact infection, was less obvious. The appearance of antihaemagglutinins after the contact period was accompanied by an unappreciable increase in the virus-neutralizing activity of its serum, and the intramuscular booster dose evoked an equivocal response.

The antibodies to influenza B virus in sera obtained from four herds, each affected by an influenza-like outbreak 4–6 months before blood sampling, support the assumption that influenza B virus is able to infect swine. Although the HI titres ranging from 1 : 10 to 1 : 40 might be regarded, at least partly, as nonspecific, due to periodate-resistant factors, the 1 : 160 HI titre and 1 : 512 neutralization titre in a particular case, appear to be convincing. It should be added that before periodate treatment almost all the serum samples under study inhibited the influenza B virus.

The age distribution of the incidence of antibodies was not characteristic. In herd V the incidence was higher (42.5 per cent) in the animals over one year of age than in the piglets under one year (12 per cent). Among the youngest piglets 33 piglets three and a half months of age (in herd VII), 18 per cent were positive.

The present results are suggestive of the susceptibility of the domestic pig to influenza B virus. Final conclusion needs, however, a direct evidence of viral reproduction in the respiratory tract.

As regards the anti-PR8 activity of 11 sera, we supposed that pre-treatment with KIO_4 , although performed according to a widely accepted prescription, was insufficient for the complete destruction of nonspecific inhibitors, or else, the antibodies had been evoked by a heterologous influenza A virus. Although five of the PR8-positive sera (titres from 1 : 8 to 1 : 32) were negative to all the other strains, a possible role of nonspecific inhibitors that had resisted KIO_4 treatment cannot be excluded.

The presence of antihaemagglutinins to swine-1 virus in the sera of pigs under one year of age in herd VI, a herd free of other antibodies, suggests that the late-autumn outbreak in that farm might have been due to a virus related to swine-1. In groups I and III the swine virus was inhibited by sera of older animals.

The inhibitor-resistant mutant of Singapore 1/57 was inhibited by a single serum which inhibited the other two influenza A strains as well.

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Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója

Műszaki szerkesztő: Farkas Sándor

A kézirat nyomdába érkezett: 1967. VII. 10. — Terjedelem: 7,50 (A/5) ív, 19 ábra, 2 melléklet

67,64084 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

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IMMUNOLOGY

An Official Journal of the British Society for Immunology

Editor: L. E. GLYNN

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ACTA MICROBIOL. ACAD. SCI. HUNG.

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STUDIES ON THE ANTIGENIC STRUCTURE OF MYCOBACTERIA

II. ANTIGENIC STRUCTURE AND LIPID FRACTIONS OF MYCOBACTERIUM AVIUM AND AVIUM-LIKE STRAINS

By

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(Received March 14, 1967)

Summary. The antigenic structure of, and the lipid fractions in, *M. avium* and avium-like strains have been studied. In *M. avium* strains 9, in avium-like strains 8 to 10 antigenic components were determined by gel-precipitation and immunoelectrophoresis. On gel-precipitation test the two groups of mycobacteria showed identical antigenic patterns, and immune sera for *M. avium* strains could be completely absorbed by avium-like strains. All examined strains were closely related antigenically.

Thin-layer chromatograms developed in iodine vapour showed that, except for one strain, *M. avium* and avium-like strains differed only in the R_f values of their lipid fractions. Untreated chromatograms examined in U. V. light revealed in avium-like strains a fraction showing bright green fluorescence. This fraction was not demonstrated in *M. avium* cultures.

In the last decades a number of mycobacteria were isolated which differed from typical strains in morphological, cultural, pathogenic and chromogenic properties. These cultures could not be classified with the classical methods. Most authors regarded these "atypical mycobacteria" as variants and not as individual species. Neither the pathogenicity nor the systematic position of these organisms has so far been elucidated.

Several authors [4—6, 8, 12, 13, 17, 21] described strains showing some minor differences from *M. avium*. These cultures were termed as "avium-like" organisms. More recently RUNYON [15] classified atypical mycobacteria into 4 groups characterized by pigment production, chromogenicity and growth-rate: I, photochromogenic; II, scotochromogenic; III, non-chromogenic; IV, rapidly growing mycobacteria. *M. avium* strains belong to group III.

BÖNICKE [1] demonstrated that members of RUNYON's group III were biochemically uniform in that they decomposed pyrazineamide and nicotinamide. These results were confirmed by SZABÓ [19] who showed in addition that there was no biochemical difference between *M. avium* and avium-like strains.

MEISSNER's [10] examinations revealed that *M. avium* strains were more virulent than avium-like strains. The difference, however, was not sufficient to allow the latter to be classified into a separate subgroup and therefore MEISSNER recommended the name "less virulent *M. avium*" for avium-like organisms.

The antigenic structure of atypical mycobacteria is even less known. JENSEN *et al.* [7] divided mycobacteria into 7 antigenic groups on the basis of the Schulz-Dale phenomenon. In this way no difference was revealed between *M. avium* and avium-like strains and they were thus classified into the same group.

There are different views as to the origin of atypical mycobacteria. In some authors' opinion [11, 16] saprophytic bacteria of plants may become parasitic in animals. These mycobacteria, which are not necessarily pathogenic for the animal, may be conveyed by milk and may cause allergy in man. According to WEISZFEILER [22], pathogenic mycobacteria may assume saprophytic properties under the influence of certain environmental effects. In other authors' opinion [23] antituberculous drugs may play a part in the development of atypical mycobacteria. LÁNYI [9] assumed that such mycobacteria might be excreted by the tuberculous patient in which they had been induced by an as yet unknown factor of the host.

This paper presents studies on the antigenic structure of *M. avium* and avium-like strains and the lipid fractions detectable in these organisms.

Materials and methods

Strains. *M. avium* strains: Bp. GH, Bp. 50, Bp. 65, Bp. 87, Bp. Z, Brno 7049, Brno 7063, Brno 7086, Brno 7109, Brno 7133, Chester; avium-like strains: 210, 303, 314, 345, 319.

Immune sera were prepared with *M. avium* strains B. Ch, Chester, Brno 7049, Brno 7109, and avium-like strains 303, 345. Rabbits were immunized with 4 week Sauton cultures emulsified in Freund adjuvant as described in a preceding paper [20].

Antigens were prepared by ultrasonic treatment of 8 week Sauton cultures [20].

Antigenic structure was examined by agar gel precipitation and immunoelectrophoresis. OUCHTERLONY's method [14] using agar gel prepared with pH 7.4 veronal buffer was employed. After pipetting the antigens and immune serum into the wells, the plates were incubated at 37°C for 48 hours. The plates were inspected daily; final readings were made on the sixth day.

The immunoelectrophoretic method of GRABAR and WILLIAMS [3] was somewhat modified [20]; prior to being pipetted into the troughs, the immune sera, similarly to the antigens, were mixed into agar gel cooled previously to a suitable temperature. The number of detectable antigenic factors was determined and the antigens were denoted according to their electrophoretic mobility with letters of the alphabet [20].

Extraction of lipids. FOLCH's method [2] was used. Five-hundred mg of bacteria were suspended in 4 ml chloroform-ethanol mixture (1 : 1). After heating to the boiling point, cooling and adding 2 volumes of chloroform, the suspension was overlaid with 15 ml distilled water. After 24 hours the organic solvent phase containing pure lipids was concentrated to 1/4 of its original volume.

Thin-layer chromatography. Three g of Kiesel-gel G were suspended in 15 ml distilled water and layered on an 8 by 18 cm defatted glass plate. The layer was inactivated at 120°C for 15 minutes. Ascending chromatography was performed in a closed glass vessel. Butanol: acetic acid : water (120 : 30 : 50) was used as solvent. The result was read first in U. V. light, then after development in iodine vapour.

Results

1. Gel-precipitation test and immunoelectrophoresis. With OUCHTERLONY's gel-precipitation method 9 antigenic factors were revealed in *M. avium* strains isolated in Budapest (Fig. 1).

The Brno strains of *M. avium* contained 9 antigenic factors (Fig. 2), which, according to gel-precipitation test, were identical with the antigens of the Budapest strains.

Avium-like strains differed from one another in the number of detectable antigenic factors. Strain 303 showed 10 precipitation lines with the

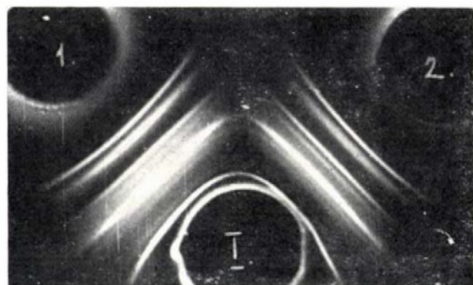


Fig. 1. Agar gel-precipitation. I = serum, *M. avium* Bp. GH; 1 = antigen, *M. avium* Bp. GH; 2 = antigen, *M. avium* Bp. 65

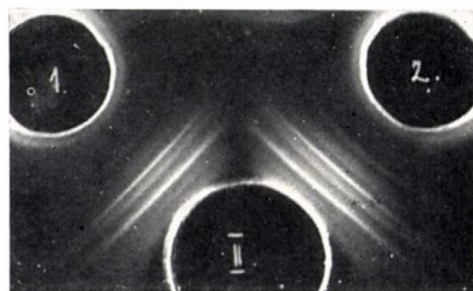


Fig. 2. Agar gel-precipitation. II = serum, *M. avium* Brno 7049; 1 = antigen, *M. avium* Brno 7049; 2 = antigen, *M. avium* Brno 7109

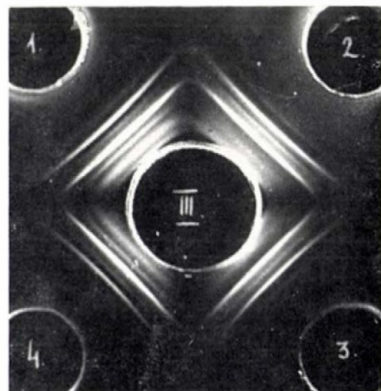


Fig. 3. Agar gel-precipitation. III = serum, avium-like 345; 1 = antigen, avium-like 345; 2 = antigen, avium-like 210; 3 = antigen, avium-like 319; 4 = antigen, avium-like 314

homologous serum; strain 314 contained 8 (Fig. 3), each of the other strains contained 9 antigenic factors. The gel-precipitation test indicated that avium-like strains were identical in antigenic pattern with the Budapest and Brno strains of *M. avium* (Fig. 4).

Testing of *M. avium* strain Chester with the homologous serum revealed 10 components.

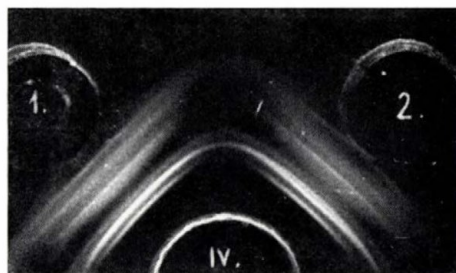


Fig. 4. Agar gel-precipitation. IV = serum, avium-like 303; 1 = antigen, avium-like 345; 2 = antigen, *M. avium* Bp. 50

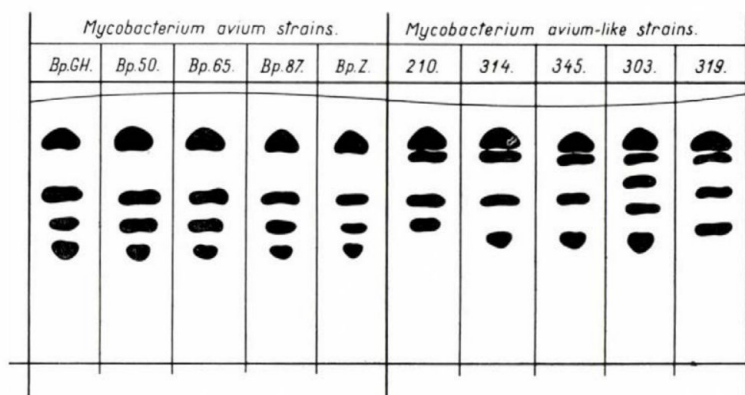


Fig. 5. Chromatograms of lipid extracts developed in iodine vapour

In addition to the similarity in gel-precipitation pattern, the close antigenic relationship between *M. avium* and avium-like strains was indicated by the fact that *M. avium* sera were completely absorbed by avium-like antigens. In immunoelectrophoretic experiments *M. avium* and avium-like antigens exhibited the same mobility.

2. *Chromatography.* The lipids extracted from living *M. avium* and avium-like bacteria are shown in Fig. 5. On chromatograms developed in iodine vapour each *M. avium* and each avium-like strain contained 4 lipid fractions. The corresponding fractions differed only quantitatively and in their R_f value. Strain 303, which yielded 5 lipid fractions, was an exception.

There was a significant difference when the chromatograms were examined in U. V. light without any treatment (Fig. 6). Extracts of *M. avium* strains yielded 3 to 5 spots with bluish fluorescence. In avium-like strains 3 or 4 lipid fractions were observed, one of which ($R_f = 0.77$) was characterized by bright green fluorescence. This fraction was present in all avium-like strains, but not in *M. avium* strains.

The green fluorescent lipid fraction was not shown when killed avium-like bacteria were extracted instead of living ones.

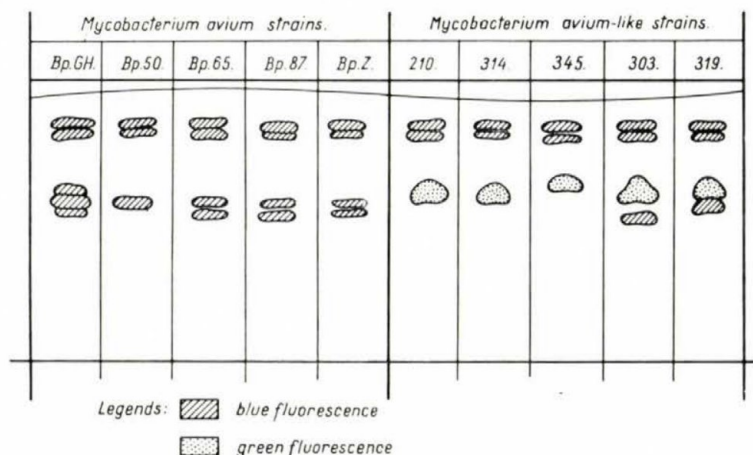


Fig. 6. Chromatograms of lipid extracts examined in U. V. light

Discussion

The cultural and chromogenic properties of *M. avium* and avium-like strains have been demonstrated by many investigators. BÖNICKE [1] showed that these organisms are identical in biochemical reactions. Some small differences in pathogenicity were not sufficient to serve as a basis of classification.

From the present studies the following conclusions have been drawn.

(1) The examined *M. avium* and avium-like strains contained 8 to 10 antigenic components. In gel-precipitation tests *M. avium* and avium-like strains showed identical antigenic patterns.

(2) Thin-layer chromatography of lipid extracts revealed significant differences between the two kinds of organism. When developed in iodine vapour the chromatograms of *M. avium* and avium-like strains differed only in R_f value of the lipid fractions. When untreated chromatograms were examined in U. V. light, avium-like strains showed the presence of a lipid fraction with bright green fluorescence. This constituent was not demonstrated in *M. avium* strains. This finding offers a reliable method for distinguishing between the antigenically closely related *M. avium* and avium-like cultures.

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THE EFFECT OF THE PHYSICO-CHEMICAL PARAMETERS OF STREPTOCOCCUS PYOGENES CULTURES ON THE FORMATION OF O-STREPTOLYSIN

I. THE ROLE OF GLUCOSE CONCENTRATION AND pH IN THE FORMATION OF O-STREPTOLYSIN

By

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Summary. The degree and rate of pH changes has been found a suitable indicator of the degree and rate of glucose consumption of streptococcus cultures.

Among culture media with glucose contents ranging from 0.12 to 3.0%, the highest cell count was obtained with the 1.0% medium, upward or downward deviations from this concentration had an adverse effect on the highest cell count obtained.

Specific growth rate in the exponential phase was limited if the glucose concentration was below 0.5%, but was not affected at higher concentrations.

In respect of O-streptolysin formation, a glucose concentration of about 1.0% and neutral pH, maintained during the entire cultivation period, were found to be optimal. If glucose concentration was above or below 1.0%, O-streptolysin formation was reduced to a greater extent than the resulting total cell count.

The present study had the aim to determine the relationship between the nutritive substances consumed by streptococci and the changes of physico-chemical parameters, with special emphasis on the optimum circumstances of O-streptolysin (OSL) formation.

The first pertaining report paid no attention to the relationship between the glucose concentration of the culture medium and OSL formation. The culture media in widest use [4, 9, 10, 13] contain 0.2% glucose regardless of the fact that MUELLER and KLISE [8] in 1932 already employed higher glucose concentrations and pH correction for mass cultivation of streptococci. BERNHEIMER and PAPPENHEIMER [2] raised the obtainable cell count tenfold by increasing the glucose content to 40% and reneutralizing the culture every hour. Among others, KARUSH *et al.* [5] studied the multiplication rate and cell count of streptococci as well as the glucose and pH limits under continuous dilution cultivation conditions. However, OSL formation received no special attention in the course of these and other investigations. BERNHEIMER [1] assumed "OSL is formed automatically, as a consequence of growth, and does not require extranutritional factors".

We have therefore studied whether the degree of OSL formation depended solely on the cell count or was affected in addition by the glucose concentration and pH of the culture.

Materials and methods

(1) *Bacterial strain.* We performed our tests with an H46A *Streptococcus pyogenes* strain of the C group. This strain was recommended by CHRISTENSEN [3] for streptokinase production but we have found [14] that its OSL-forming capacity surpassed that of the international standard strains used so far.

(2) *Culture medium.* KALBAK's [4] medium was used with the modification that instead of neopeptone we used Lactamine, an enzyme digested casein produced by our Institute, at a concentration of about 2%, so as to keep the alpha N value of the medium at 1.1–1.2 mg/ml.

The concentration of glucose was changed in the experiments between 0.12 and 3.0% as detailed below.

(3) *Preculture.* The lyophilized strain was grown on blood agar plates and isolated colonies were inoculated into 10 ml of KALBAK's medium, where a threefold passage was carried out in a manner that cultures of 4 to 6 hours were transferred every time.

(4) *Cultivation* was carried out in 500 ml Erlenmeyer flasks each containing 300 ml of medium, at a temperature of 37°C. The media were inoculated with 1/10 volume of 6-hour pre-cultures. In the course of cultivation, 5–10 ml samples were taken every hour for measuring optical density (OD), haemolytic activity, pH and glucose concentration.

(5) *pH measuring* was done by means of an electric pH meter (Radiometer).

(6) *Cell count.* OD was estimated by zeroing for the sterile culture medium, by means of FEK Stanco photoelectric colorimeter. 1.5 OD measured with the apparatus corresponded to 100 O. U. of the NIH turbidity standard which according to the nomogram of KOCH and KAPLAN [6] corresponds to 6.4×10^9 per ml of streptococci.

(7) *Haemolytic activity.* The samples were centrifuged immediately and reduced with 0.4% NaHSO₃. Haemolytic activity was determined in 1.5 ml total volume, of which 1.0 ml was diluted culture fluid, and 0.5 ml was 2% washed sheep erythrocyte suspension. As titre was regarded the dilution in the tube that produced 50% haemolysis observable with the naked eye after 45 minutes incubation in a 37°C water bath; its reciprocal was used as the Haemolytic Unit (H. U.).

(8) *Glucose determination* was performed with SUNDERMAN's [12] method, with glucose oxidase (BDH) photometrically.

Results

In the first series of experiments, we studied how a regular readjusting to the starting level of the decreased pH and glucose concentration affects the cell count and OSL titre as compared to untreated controls. The first series (marked A, B, C) contained 0.25% glucose — *i. e.* a quantity approximating the 0.2% content of KALBAK's medium; the second series (marked D, E, F) contained 1.0% of glucose. From the 3rd to the 8th hour, samples were taken from each flask every hour. On the basis of their pH, cultures B and E were readjusted with 20% NaOH to pH 7.1–7.4, and in cultures C and F the glucose concentration was readjusted by adding 50% glucose in a 1 : 1.1 ratio of the volume of NaOH required for readjusting the pH. Cultures A and D were left untreated to serve as controls. No other adjustments were made after the 8th hour, only a final sample was taken from each flask at 24 hours.

Results are shown in Table I. In cultures A and B, cell count and OSL titre reached their maximum in the 7th hour; a moderate decrease was noted in the 8th hour, a marked one in the 24th hour, with a simultaneous light alkalescence of the culture. In culture C, glucose concentration was readjusted to 0.25% in the 6th, 7th and 8th hours, but it fell to 0 again in one hour,

Table I

Characteristic data of *Str. pyogenes* H46A cultures with 0.25 and 1.0% glucose concentration
Correction of pH or that of pH and glucose concentration

Labelling of cultures	Medium at inoculation		From the 3rd hour maintained at		Glucose mg/ml consumed during 8 hours	Maximal optical density	Maximal haemolytic units/ml	H. U.
	pH	glucose %	pH	glucose %				O D
A	8.1	0.25	—	—	1.25	0.97	98*	101
B	8.1	0.25	7.1—7.4	—	1.75	1.02	98*	96
C	8.1	0.25	7.1—7.4	0.2—0.3	2.34	1.20	125	104
D	8.1	1.0	—	—	4.80	1.20	125	104
E	8.1	1.0	7.1—7.4	—	9.80	1.50	225	150
F	8.1	1.0	7.1—7.4	0.9—1.1	17.90	1.60	416	260

Note: the maximum OD and H. U. values were achieved at the 8th hour, but in the cultures signed* at the 7th hour.

indicating thereby that the glucose requirement of the cells engaged in intense metabolism was higher than the amount of glucose available to them. At the end of cultivation, the culture became slightly alkaline.

In control culture D (see Fig. 1), the initial 1.0% glucose decreased to 0.44%, the pH to 5.2, by the 8th hour. Cell count and OSL titre increased until the 7th hour. Exponential multiplication was stopped in this case prob-

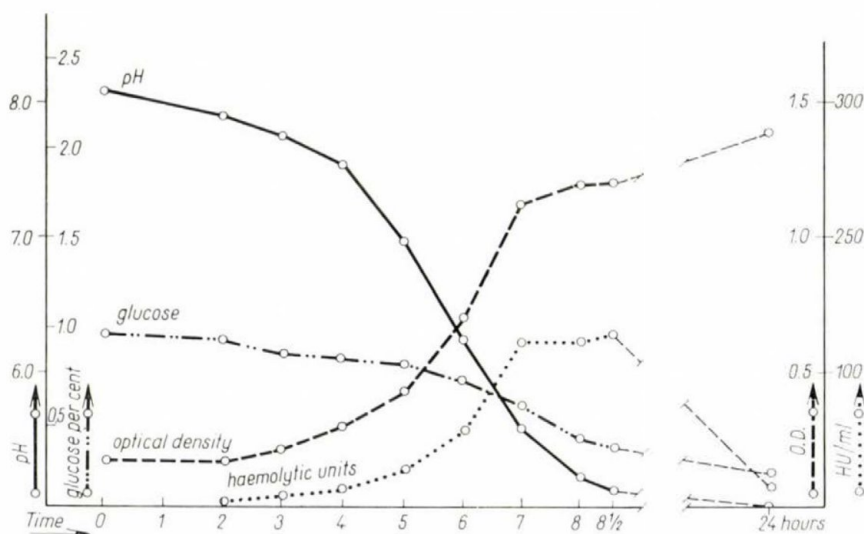


Fig. 1. Glucose concentration, pH, optical density and haemolytic activity in a stationary culture of *Str. pyogenes* H46A

ably by the culture becoming acidic, as it still contained some 0.5% of glucose. In culture E no glucose was found 1/2 hours after inoculation (see Fig. 2). Increase of the OSL titre, like in culture D, proceeded until the end of the exponential phase, but it was not the acidification that determined the end of the exponential phase in this case — as we had regularly readjusted the pH to above 7.0 — but the decrease of the glucose content below 0.1%. This means that the culture consumes 1.0% glucose fully if the pH is maintained above



Fig. 2. Glucose concentration, pH, optical density and haemolytic activity in a stationary culture of *Str. pyogenes* H46A, with the pH corrected to neutral between the 6th and 9th hour

the neutral point, as a result of which higher cell counts and higher OSL titres can be obtained. If the pH and the glucose concentration are corrected simultaneously — as in culture F — (see Fig. 3) the culture is able to consume as much as 17.9 mg/ml, that is nearly 2.0% of glucose, which causes a slight rise in the cell count, but a considerable one in the OSL titre if compared with culture E. The increase of the OSL titre, as in the case of cultures D and E, only proceeds until the end of the exponential phase, but it is obvious that the end of this phase was determined not by an extreme decrease of pH and glucose, but by other limiting factors.

It was not possible to establish from the above data whether there existed a threshold concentration of glucose above which the germ count and the OSL titre would not have increased further. In the second series we therefore performed cultivations in which the glucose concentrations had been set at 0.12, 0.25, 0.5, 1.0, 1.5, 2.0 and 3.0%, respectively. From the 2nd to the 8th hour of cultivation, we took samples every hour and readjusted the pH to about 8, and the concentration of glucose to the initial values. (For illustra-

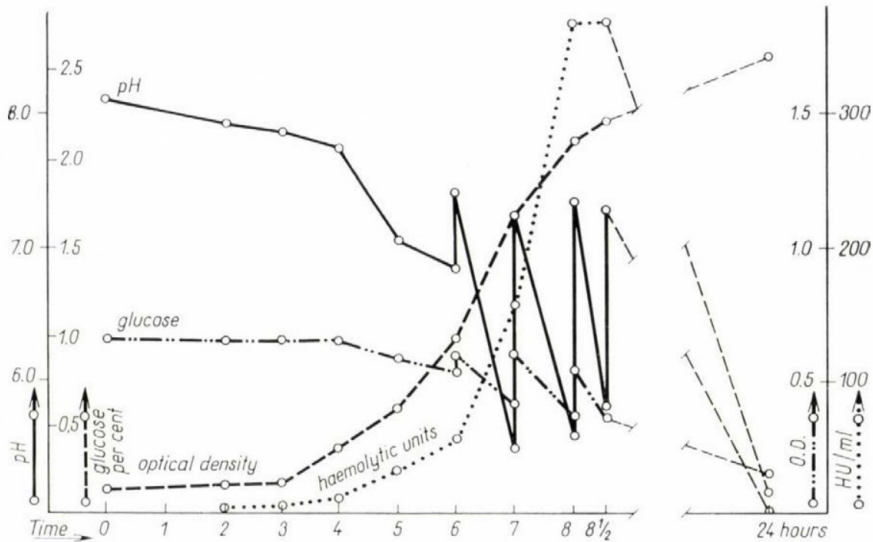


Fig. 3. Glucose concentration, pH, optical density and haemolytic activity in a stationary culture of *Str. pyogenes* H46A, with the pH corrected to neutral and the glucose concentration to 1.0% between the 6th and 9th hour

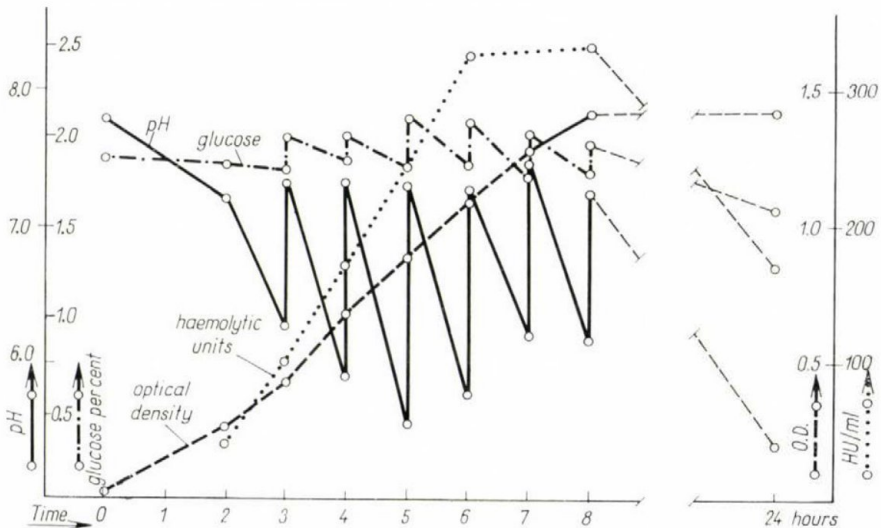


Fig. 4. Glucose concentration, pH, optical density and haemolytic activity in a stationary culture of *Str. pyogenes* H46A with 2.0% glucose content, with the pH corrected to neutral, and glucose concentration to 2% between the 3rd and 8th hour

tion, the parameters of a culture containing 2.0% glucose are shown in Fig. 4.) The changes in optical density of the seven parallel cultures are shown in the next two figures, in dependence on time in Fig. 5 and on the glucose concentration in Fig. 6. The principal data for the exponential phase between

the 3rd and 6th hour, are shown in Table II. From Figs 5 and 6, and Table II, it appears that the glucose concentrations of 0.12% and 0.25% were limiting both the obtainable cell count and the rate of multiplication. The optimum cell count was observed with 1.0% glucose, but nearly identical results were obtained with 0.5% glucose. Thus saturation with glucose of KALBAK's medium

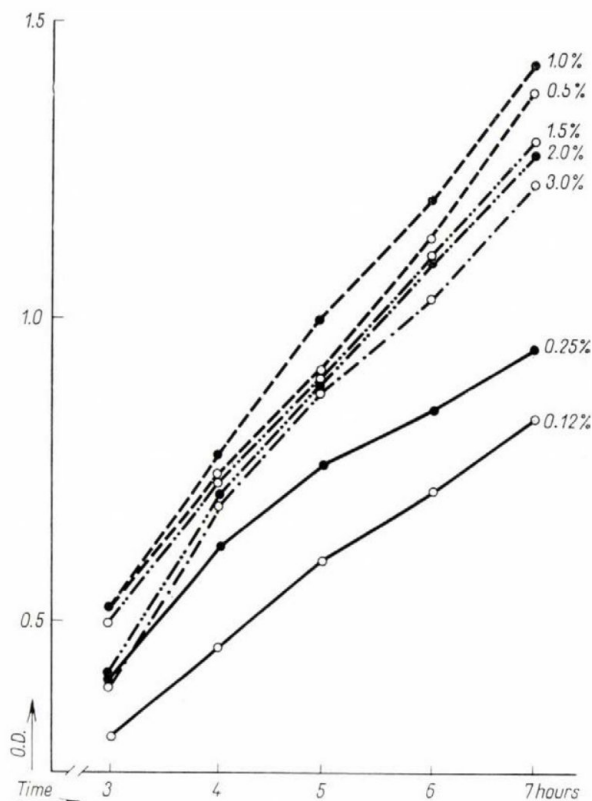


Fig. 5. Optical density in seven cultures of *Str. pyogenes* H46A with 0.12, 0.25, 0.50, 1.0, 1.5, 2.0 and 3.0% glucose content, respectively, between the 3rd and 7th hour, with the pH and glucose concentration corrected to the initial level

is between the continuously maintained concentrations of 0.5 and 1.0%. At and above the saturation level, the streptococci consumed from 1.0 ml of culture medium 3 mg per hour of glucose. However, raising of the glucose content above the saturation level affected adversely the optical density. While a total cell count of 75.0 O. U. was obtained with 1.0% glucose, the cell count obtained with 3.0% glucose was only 60.0 O. U. by the 6th hour. Thus, the total cell counts obtained by the 6th hour rose gradually with 0.12 to 1.0% of glucose, and decreased gradually with 1.0 to 3.0% of glucose. This order

of total cell counts had, however, been established by the end of the 3rd hour, during the lag phase. Concerning the rate of multiplication, the cultures below and above the glucose saturation level differed from one another in the exponential phase. After the 3rd hour, there was no difference in the rate of multiplication between cultures containing 0.5% or more of glucose, and the

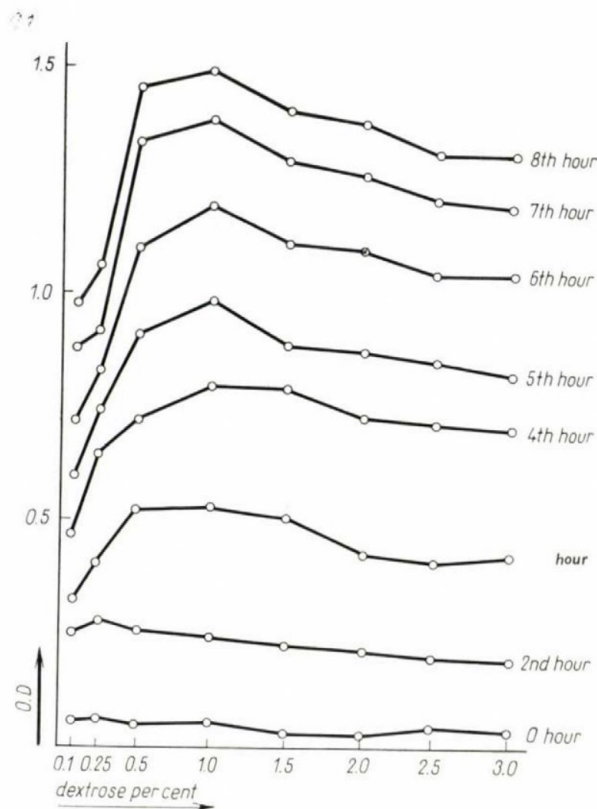


Fig. 6. Optical density in eight cultures of *Str. pyogenes* H46A as a function of the glucose concentration, with the pH and glucose concentration corrected to the initial level, between the 3rd and 7th hour

germ count curves ascended in parallel, but the rate of multiplication of cultures below the saturation level was reduced in the exponential phase, too. Finally, if the seven cultures were compared in respect of the efficiency of glucose utilization, there appeared no difference, as 5.5 O. U. microbes were formed in the exponential phase on the consumption of 1 mg of glucose, whether the cultures contained 0.25% or 3.0% of glucose.

As to the efficiency of glucose utilization, a qualitative difference appeared between bacterium multiplication and OSL formation. Namely,

while multiplication was essentially the same at various glucose concentrations, OSL formation was four times more intense at 1.0% than at 0.25% glucose concentration and twice as much as at 3.0%.

The OSL titres in dependence on glucose concentration are illustrated in Fig. 7. From a comparison of Figs 6 and 7 it appears that the maintenance

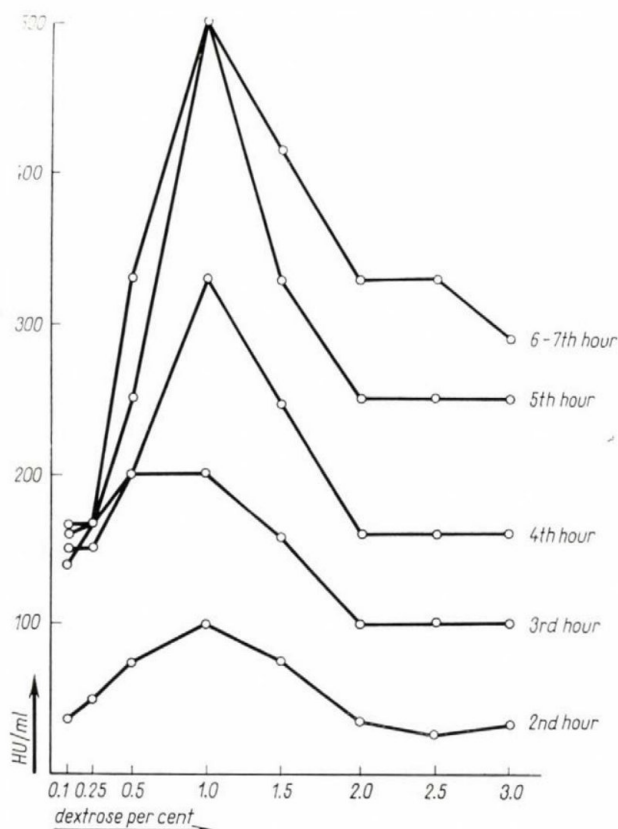


Fig. 7. Haemolytic activity in eight cultures of *Str. pyogenes* H46A, as a function of the glucose concentration, with the pH and glucose concentration corrected to the initial level between the 3rd and 7th hour

of the glucose level at 1.0% was strikingly more favourable for OSL formation than for the total germ count.

Cultivation under identical conditions as with culture F was repeated ten times; the maximum of haemolytic activity reached 416–500 H. U./ml, the maximum values of optical density were between 1.56 and 1.65, corresponding to a cell count of $6.6-7.0 \times 10^9$ per ml according to KOCH and KAPLAN's [6] nomogram. The maximum cell counts and OSL titres were obtained in

Table II

Glucose consumption of Str. pyogenes H46A culture at different glucose levels and its utilization in producing cell count (O. U.) and haemolytic activity (H. U.) in the exponential phase

Glucose concentration, per cent	0.12	0.25	0.50	0.1	1.5	2.0	3.0
Quantity of consumed glucose mg/ml in the exponential phase per one hour	1.51	1.81	2.41	3.09	3.06	2.92	2.92
Turbidity (O. U.) in 3rd hour	7.5	17.5	22.5	22.0	20.0	17.0	12.5
Turbidity (O. U.) in 6th hour	37.5	47.5	65.0	75.0	62.5	62.0	60.0
Produced O. U. between the 3rd and 6th hour (difference)	30.0	30.0	42.5	53.0	42.5	45.0	47.5
Haemolytic units/ml in 3rd hour	160	160	200	200	160	160	150
Haemolytic units/ml in 6th hour	165	200	333	500	333	333	292
Produced H. U. between the 3rd and 6th hour (difference)	5	40	133	300	173	173	142
Consumption of 1 mg glucose in O. U. during 1 hour in the exponent	6.6	5.5	5.8	5.7	4.6	5.1	5.4
phase resulted } in H. U.	1.1	7.3	18.3	32.3	18.8	19.7	16.2

the 6th — 8th hour; the time of cultivation depended on the length of the lag phase, the latter on differences in the inocula.

Discussion

Our results have confirmed the conclusion [2, 3, 8] that in certain streptococcal culture media a glucose concentration of about 0.2% acts as the primary limiting factor of the multiplication of streptococci. If glucose is applied in excess, the secondary limiting factor appears to be the rapid acidification of the culture to pH 4.0—5.0, since the streptococci transform 87—95% of the glucose into acidic products, especially lactic acid [11, 15]. If the acidic metabolic products formed in this way are repeatedly neutralized with NaOH in the course of cultivation, multiplication proceeds until it is again arrested by further limiting factors.

In our experiments, the quantity of NaOH used for re-neutralization and the rate of glucose consumption were proportional to the quantity of glucose present and its rate of utilization. The quantitative aspect of this relationship is expressed by the ratio 1 M glucose : 1.8 M NaOH, which means that the acidic decomposition products of 1 ml of 50% glucose are neutralized by 1 ml of 20% NaOH. We found this ratio to be valid mainly in cases where the glucose level was 1.0% and the pH between 6.9 and 7.3; at glucose levels and pH values different from these, the ratio was somewhat modified. From glucose concentrations between 1.0 and 3.0%, the streptococci consumed not

more than 3 mg per ml per hour in the exponential phase, thus the quantity of NaOH required for neutralization was 6 ml per litre per hour. In the literature available to us sufficiently detailed data for comparative calculations have been found only in CHRISTENSEN's paper [3]. CHRISTENSEN used the same H46A strain for streptokinase production, but he supplied the energy source by adding to the culture medium glucose of 3.4% technical purity in one dose, and needed in this case 4 to 10 ml of 20% NaOH per litre per hour for neutralization, which is equivalent to the consumption of 2 to 5 mg of glucose.

The next point was to determine the glucose saturation value (K_s) of the medium with identical inocula and under identical conditions. It was found to be 1.0% or somewhat less. Lower levels limited, higher ones did not affect the growth rate of about 60 minutes in the exponential phase. A surplus cell count corresponding to 15.5 O.U. per hour per ml on the average was formed in cultures saturated with glucose. Although at the end of the exponential phase, in the 7th–8th hour there appeared differences in the maximum cell count of cultures containing 1.0 to 3.0% glucose, in the paradoxical manner that raising the glucose level above 1.0% resulted in a lower maximum of cell count. This was in accordance with the data of KARUSH *et al.* [5], who attained maximum turbidity with 0.04 M (= 0.72%) glucose, and found that on increasing the concentration maximum turbidity decreased.

The cause of the differences in the maximum cell counts must be sought in the events of the lag phase. Cell counts identical at inoculation showed differences in the culture media containing 1.0–3.0% glucose in 3 hours, at the end of the lag phase, and since in the exponential phase the multiplication rates were identical it were actually the differences developed by the end of the 3rd hour that persisted up to the end of the exponential phase.

According to MONOD's [7] formula $\mu = \mu_{\max} \left(\frac{S}{K_s + S} \right)$, the specific growth rate (μ) is not limited by the substrate concentration (S) above the saturation constant value of the substrate (K_s). Our results have confirmed this. However, this conclusion cannot be interpreted in a manner that any amount of "safety surplus" can be applied above the saturation level of nutritive substances indispensable for growth, as not only a saturation threshold value, but an optimum value as well may exist for them. For instance a glucose concentration higher than the optimum does not affect specific growth in the exponential phase, but owing to the inhibition produced in the lag phase the maximum germ count is lower nevertheless, and the OSL titre is still much lower owing to other mechanisms.

Biologically active substances produced by microbes are usually the products of living and reproducing organisms, and the assumption seemed obvious that if a higher germ count is obtained, the special products of the microbes will be formed at accordingly higher concentrations. Yet, we consid-

ered it worth while to re-examine this assumption, which was formulated in respect of OSL by BERNHEIMER [1]. If we compare the maxima of cell counts and OSL values observed in our culture, it appears that slight differences in cell count are accompanied by marked differences in OSL titre. We therefore concluded that the OSL-forming enzyme system was in some relationship with the self-reproducing system of the microbe, and that it was sensitive to factors of which the self-reproducing system is able to render independent itself to a greater extent. For example, it may be a substantial difference from the point of view of energetics whether pyruvic acid is transformed into lactic acid, or broken down in oxidative ways. In principle, the possible ways of transformation may be determined by the actual concentration of glucose, and it might be in this way that it affects the degree of OSL formation. Further research in this direction might modify the present interpretation of the relationship between glucose concentration, multiplication and OSL formation.

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THE EFFECT OF THE PHYSICO-CHEMICAL PARAMETERS OF STREPTOCOCCUS PYOGENES CULTURES ON THE FORMATION OF O-STREPTOLYSIN

II. THE ROLE OF REDUCING AGENTS IN THE FORMATION OF O-STREPTOLYSIN

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(Received March 14, 1967)

Summary. The effect of sodium thioglycolate, sodium bisulphite, sodium dithionite, glutathione, methionine, thiamine, cystine, cysteine and ascorbic acid on the growth and O-streptolysin-producing ability of the group C *Streptococcus pyogenes* strain H46A has been investigated under different experimental conditions. O-streptolysin production was markedly increased by the combination of cysteine + ascorbic acid and slightly by cysteine as well as the combination of cystine + ascorbic acid. The other reducing agents were ineffective in this respect. Specific growth rate of the strain was not influenced by the reducing compounds.

With the international standard group A *Streptococcus pyogenes* strains C203, S84, and Richards, used for the production of O-streptolysin, as well as with another group C strain (Tp 21) O-streptolysin production was slightly increased by the combination of cysteine + ascorbic acid, and specific growth rate was not influenced.

In relation with O-streptolysin (OSL) attention was drawn to the different reducing agents when NEILL and MALLORY [7] had demonstrated the oxygen sensitivity of OSL and the possibility of reactivation of the OSL inactivated by oxidation with sodium bisulphite reduction. Reducing agents, however, were only added to the final OSL filtrate. To the culture medium itself reducing agents were first added by SLADE and KNOX [9] who investigated the effect on OSL formation of cysteine, glutathione, sodium-thioglycolate, sodium sulphite and ascorbic acid. These authors concluded from their results that "an optimum reducing potential is required for maximum lysin production". They failed to observe OSL production during satisfactory growth on semi-synthetic culture medium containing 0.2% glucose, if reducing agents had not been added. Subsequently, ROBINSON *et al.* [8] succeeded in obtaining OSL on a culture medium containing 0.0075% cysteine, and this medium was then employed by others [1], too. Since TODD [10,11] BERNHEIMER *et al.* [2], KALBAK [4] and others prepared earlier an OSL of satisfactory titre without the use of reducing agents, it seemed interesting to study the role in OSL formation played by reducing agents after the disturbing effects of the low glucose concentration and pH had been eliminated.

Materials and methods

(1) *Bacterial strains.* The following *Streptococcus pyogenes* strains were used: H46A (group C), Tp 21 (group C), S84 (group A, type 3), C203 (group A, type 3), Richards (group A, type 3).

(2) *Culture medium.* KALBAK's medium [4] was used as described in the first part of this series [3], with the difference that glucose concentration was maintained at 1.0% throughout.

(3) *Electrode potential and pH* were estimated with two electric pH meters (Radiometer, Copenhagen). Ep signifies the value directly measured in millivolts.

(4) *Cell count, haemolytic activity and glucose concentration* were estimated as earlier [3].

(5) *Reducing agents and their applied concentration* were as follows. Sodium bisulphite (NaHSO_3), 0.1–0.5 mg/ml; sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), 0.1–0.5 mg/ml; sodium thioglycolate, 0.1–0.5 mg/ml; cystine, 0.1–0.5 mg/ml; ascorbic acid (vitamin C) 0.1–2.5 mg/ml; methionine, 0.1–3.0 mg/ml; thiamine (vitamin B_1), 0.1–5.0 mg/ml; glutathione, 0.1–5.0 mg/ml; cysteine, 0.25–5.0 mg/ml. Cysteine and cystine were examined in addition after mixing with half volume of ascorbic acid.

(6) *Cultivation.* The effect of reducing agents was investigated by two different cultivation techniques. Method I, parallel stationary cultures in 500 ml Erlenmeyer flasks, and Method II: in a single culture vessel of 10 litres volume, mounted with a controlling and recording device for pH, in successive cultivations.

Method I. 300 ml fluid culture medium is filled into Erlenmeyer flasks of 500 ml and sterile solutions of the agents to be examined are added at different concentrations. After inoculation with 10% (= 30 ml) of the same 6 hour-preculture, beginning from the second hour, samples of 5 to 10 ml are taken every hour to measure optical density (O. D.), pH, electrode potential (E_p) and haemolytic activity (H. U.). The observed pH was readjusted to neutrality by a 20% NaOH solution. Simultaneously, an equal volume of 50% glucose solution was also added to the culture. Subsequently, the pH was readjusted to neutral and glucose concentration to 1.0% every hour [3].

Method II. Cultivation was carried out uninterruptedly for 60 hours in one vessel. Having filled into the culture vessel 3.0 litres of pre-warmed culture medium, about 10% of its volume was taken as inoculum from a 6 hour culture. The temperature of the culture was kept at 37°C, and the pH between 6.9 and 7.2, and the glucose level between 0.9 and 1.1%. Changes in O. D. and in E_p value were recorded continuously. H. U. was determined in samples every 15 minutes. At the end of the exponential phase, 9/10 of the culture was drained off, and replaced by fresh culture medium, so that about 300 ml of the suspension remained in the culture vessel and this was filled up by 2.700 ml of fresh culture medium.

Simultaneously with the introduction of fresh culture medium, a sterile solution of the convenient reducing agent was added to the culture. Having attained a maximum of O. D., 9/10 of the culture was again drained off, 300 ml being left for inoculation and the system was refilled again with fresh culture medium. A reducing agent to be investigated was applied in every second culture, the intermediate one having been left as control without reducing agent. In this way if any of the reducing agents was left unused, i.e. at unchanged concentration at the end of the cultivating period, in the next culture it was present in a concentration of 1/10, and in the following culture with reducing agent at a concentration of 1/100 the original, so that its accidental late disturbing effect had been eliminated.

Results

In the first experimental series Method I was used in stationary cultures and the effect of 9 reducing agents and of two of their combinations was studied on the formation of OSL by strain H46A. These reducing agents were compounds containing an S—S or SH group: cystine, cysteine, methionine, glutathione, sodium thioglycolate; inorganic reducing agents: NaHSO_3 and $\text{Na}_2\text{S}_2\text{O}_4$; and vitamins having a reducing effect: thiamine and ascorbic acid. Each compound was examined several times, at least at three different concentrations. Results of the whole test series cannot be compared with each other

having been carried out in successive parts during several days; the effect of any one of the reducing agents could only be evaluated by comparison with the reducing agent free control of the same day.

From these preliminary investigations it could be established that OSL formation was slightly inhibited (without decreasing the cell count) by NaHSO_3 , $\text{Na}_2\text{S}_2\text{O}_4$ and cystine. On the other hand, cysteine and cystine + ascorbic acid increased formation of OSL slightly, and cysteine + ascorbic acid, considerably.

The most effective combination cysteine + ascorbic acid was examined at five different concentrations. Results are shown in Table I and Fig. 1.

In Fig. 1 it is seen that cysteine + ascorbic acid did not considerably influence the attainable cell count nor the specific growth rate, while haemolytic activity was most increased by the combination 3.0 mg/ml of cysteine + 1.5 mg/ml of ascorbic acid. The oxidation-reduction potential values and the titre of haemolytic activity as compared to each other are shown in Fig. 2.

It can be seen from Fig. 2 that the oxidation-reduction curves at different cysteine + ascorbic acid concentrations had the same character as the controls, with the difference that on increasing the concentration they still ran parallel but at a lower level. The oxidation-reduction potential of the culture attained

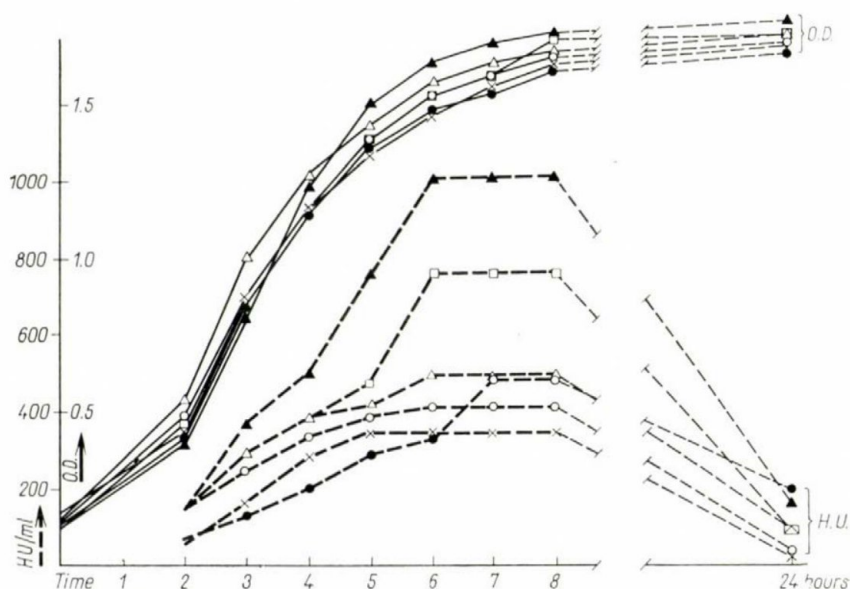


Fig. 1. Optical density and haemolytic activity in cultures containing different amounts of a cysteine-ascorbic acid mixture. Key: Blank circles: 0.5 mg/ml cysteine + 0.25 mg/ml ascorbic acid; blank triangles: 1.0 mg/ml cysteine + 0.5 mg/ml ascorbic acid; blank squares: 2.0 mg/ml cysteine + 1.0 mg/ml ascorbic acid; full triangles: 3.0 mg/ml cysteine + 1.5 mg/ml ascorbic acid; full circles: 5.0 mg/ml cysteine + 2.5 mg/ml ascorbic acid; $\times$: control without reducing mixture. O. D.: optical density H. U.: haemolytic units

Table I

Electrode potential values for *Str. pyogenes* H46A cultures with different cysteine + ascorbic acid content

Cysteine mg/ml	Ascorbic acid mg/ml	Electrode potential of the cultures	
		at inoculation	at 6th hour
0.5	0.25	-195	-120
1.0	0.5	-210	-130
2.0	1.0	-220	-170
3.0	1.5	-120	-200
5.0	2.5	-230	-260
0	0	+ 25	- 40

its lowest point when the titre of OSL reached its peak, i. e. at the end of the exponential phase. From this moment to the 24th hour the electrode potentials increased slowly, while haemolytic activity decreased rapidly.

In studies with Method II the reducing agents were applied at the concentrations found optimal in the preliminary tests, viz. at 1 mg/ml thiamine,

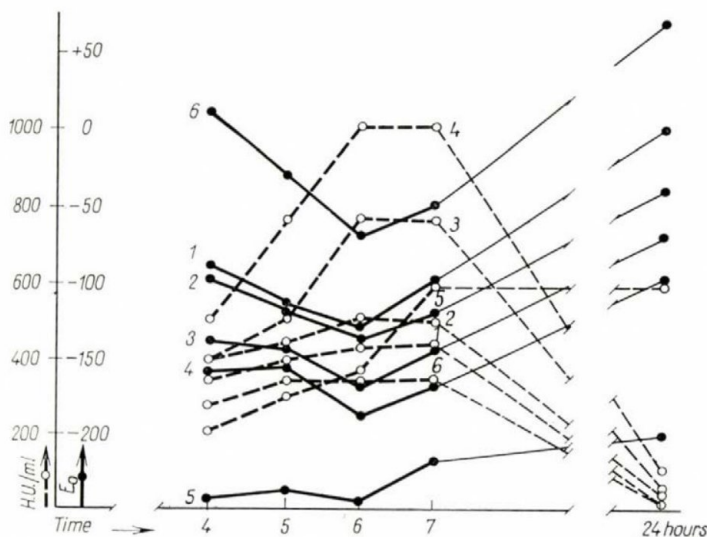


Fig. 2. Haemolytic activity and electrode potentials in the 4th through 7th and the 24th hour of cultivation, in cultures containing different amounts of a cysteine + ascorbic acid mixture. Key: Blank circles and broken lines: haemolytic units; full circles and drawn lines: electrode potential.

- 1 = 0.5 mg/ml cysteine + 0.25 mg/ml ascorbic acid
- 2 = 1.0 mg/ml cysteine + 0.5 mg/ml ascorbic acid
- 3 = 2.0 mg/ml cysteine + 1.0 mg/ml ascorbic acid
- 4 = 3.0 mg/ml cysteine + 1.5 mg/ml ascorbic acid
- 5 = 5.0 mg/ml cysteine + 2.5 mg/ml ascorbic acid
- 6 = control without reducing mixture

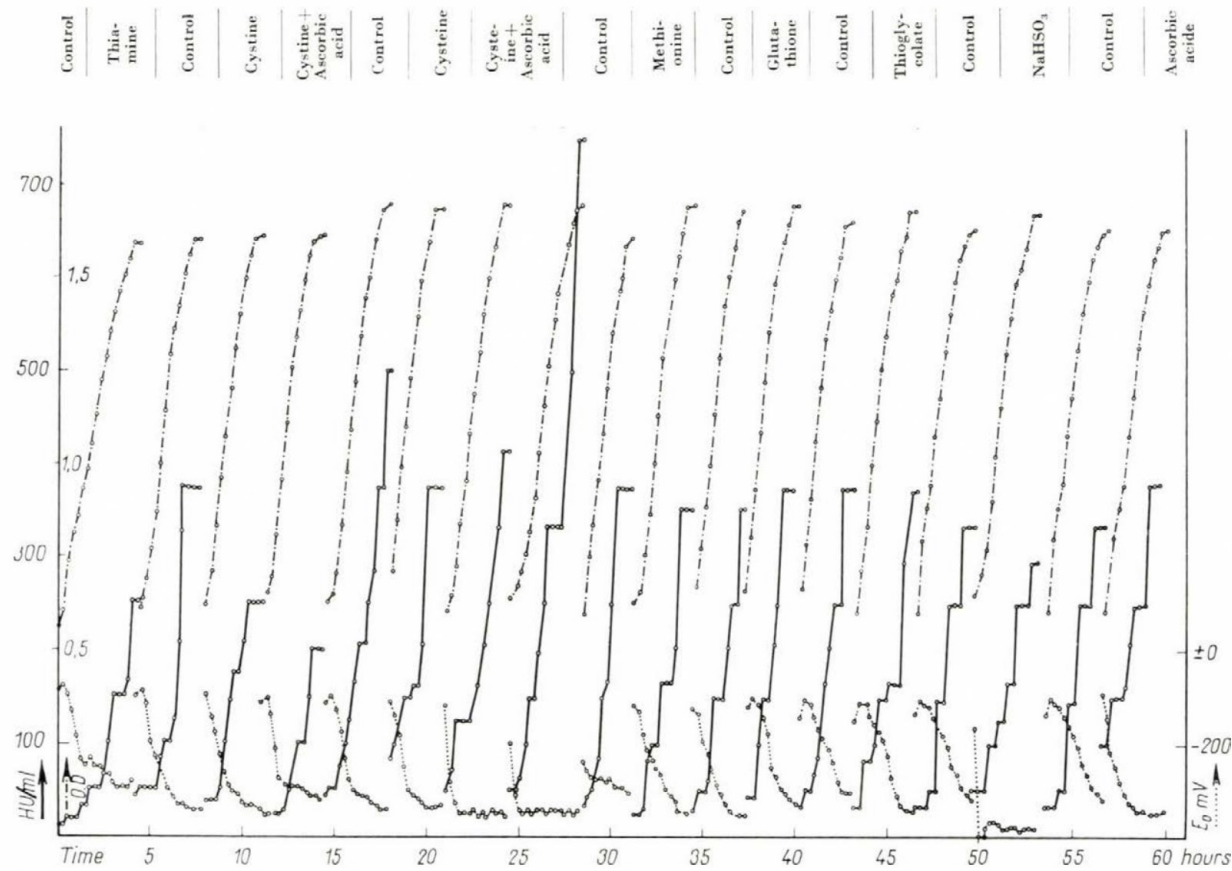


Fig. 3. Intermittent-continuous streptococcus cultivation for 60 hours, in the presence of different reducing agents, and without them.
Key: Drawn line, haemolytic units; broken line, electrode potential; dash-and-dot: optical density

0.1 mg/ml cystine, 0.4 mg/ml methionine, 3 mg/ml cysteine, 3 mg/ml cysteine + 5 mg/ml ascorbic acid, 0.1 mg/ml glutathione, 0.1 mg/ml sodium thioglycolate, 0.1 mg/ml NaHSO_3 , 2 mg/ml ascorbic acid. Sodium dithionite was not tested in this series. Results are shown in Fig. 3.

In Fig. 3 variations of the pH and glucose concentration are not indicated, since these were kept between the said limits. From the curve for O. D. it is seen that the technique employed did not disturb the rhythm of bacterial growth. This was possible because at the end of each experimental period the draining-off and filling-up of the culture took at most 4 minutes and the other parameters not having been changed, the culture was always in the phase of intensive multiplication. The O. D. curve for the particular substances also proved that specific growth rate was not affected. The electrode potential curves ran in most cases in parallel. From the oxidation-reduction curves for the other substances the curves for NaHSO_3 , cysteine, and the combination cysteine + ascorbic acid differed markedly; the latter yielded lower while 0.1 mg/ml of cystine, higher E_p values.

The differences were most conspicuous in the OSL curves in agreement with the result obtained with stationary cultures, as the combination cysteine + ascorbic acid (as compared to the controls and the other agents) increased the formation of OSL about twofold. Even the combination cystine + ascorbic acid increased the amount of OSL, although cystine alone was slightly inhibitory.

After registering that the combination cysteine + ascorbic acid had such a conspicuous stimulating effect on OSL production by strain H46A, we carried

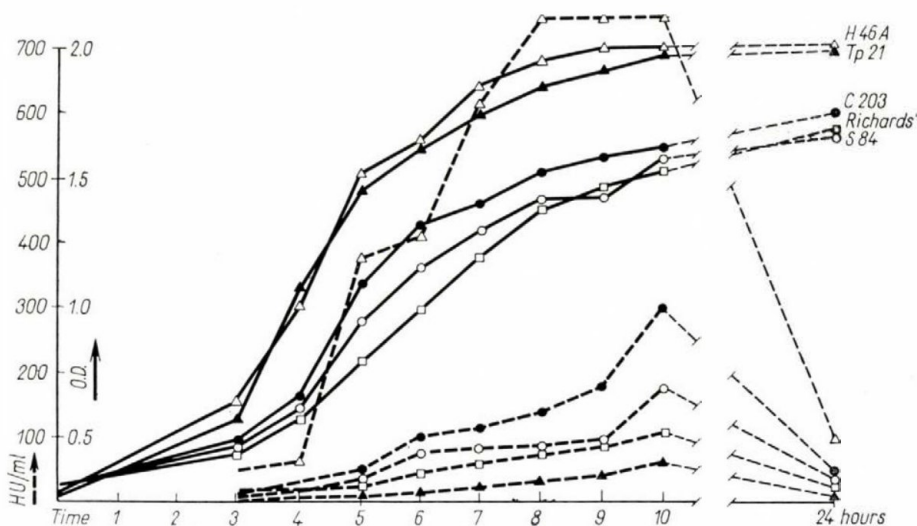


Fig. 4. Haemolytic activity and optical density in cultures of five *Streptococcus haemolyticus* strains, in the presence of 3 mg/ml cysteine and 1.5 mg/ml ascorbic acid. Key: Blank circle, S84; blank square, Richards; full circle, C203; blank triangle, H46A; full triangle, Tp21

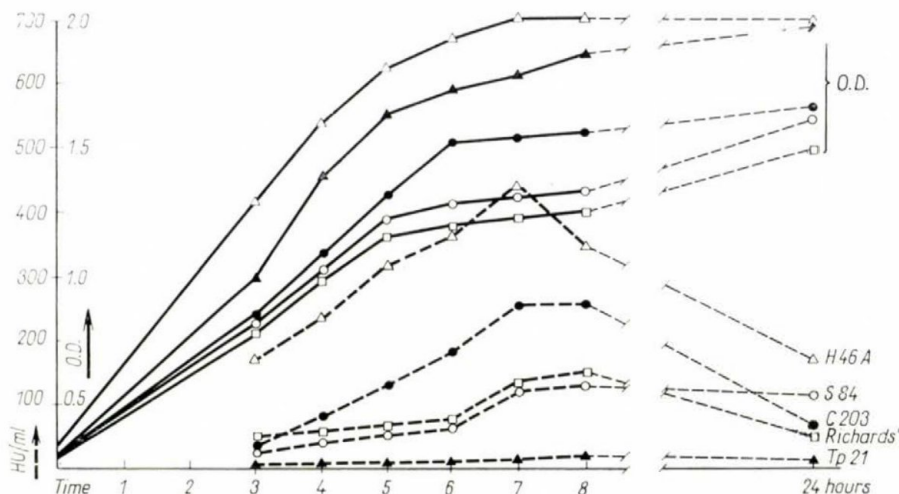


Fig. 5. Haemolytic activity and optical density in the cultures of five *Streptococcus haemolyticus* strains, without reducing mixture (controls). Key: The same as in Fig. 4

out tests to study whether the effect asserted itself also with other strains of streptococci. In the next series we examined by Method I the effect of the combination 3 mg/ml of cysteine + 1.5 mg/ml of ascorbic acid on the OSL-forming ability of group A strains of type 3, used internationally for OSL production, as well as on that of a group C strain, being weak OSL-former. The results gained with the five strains are demonstrated in Fig. 4.

As a control, cell count and haemolytic activity of these strains were checked under the same circumstances of cultivation but without cysteine + ascorbic acid. Results are shown in Fig. 5.

Thus, the combination cysteine + ascorbic acid did not have any influence on cell count and specific growth rate neither with the three group A strains, nor with the two from group C. As to haemolytic activity, in the cultures of group A strains a slight, in that of the group C strain H46A a considerable, increase was apparent, though haemolytic activity of the other group C strain changed hardly as compared with the controls. In the presence of cysteine + ascorbic acid the group A strains attained only one or two hours later their maximum OSL titre. The two group C strains reached a higher germ count in the test cultures as well as in the controls than the group A strains.

Discussion

Our previous observations [3] were indicative of a close relationship between OSL formation and the concentration of glucose *viz.* one of the alternative ways of energy release. There must be some specific effect connected

to the mode of glucose utilization which has an influence on the intensity of OSL formation without having a substantial effect on growth.

Feeling prompted by this idea we subjected to re-examination the statement of SLADE and KNOX [9] that OSL formation was closely connected with the presence of reducing agents and that the optimum of OSL formation can be characterized with "some" oxidation—reduction potential. We found that the results of SLADE and KNOX might have been influenced by the relative scarcity of the carbon source, since — according to their account — with the application of a culture medium of 0.2% glucose content the initial pH of 7.6 decreased to pH 6.7 only by the 18th hour of cultivation. In comparing these and our own results it must be taken into consideration that we worked under quite different conditions; they used a chemically defined culture medium and another *Streptococcus* strain (Str. Richards), and did not report oxidation—reduction values. Also while on their own culture medium a concentration of 0.75 mg/ml of cysteine was the optimum, we attained the best results on KALBAK's medium with fourfold amount of cysteine and half the quantity of ascorbic acid; this concentration ensured an E_p -value of about 200 mV throughout.

The present experimental conditions were set in a manner to keep the physico-chemical parameters, *i. e.* temperature, pH, glucose concentration, the ratio amino-N : C of the culture medium, etc., approximately constant during the whole time of cultivation, and to change only the reducing agent. The reducing agents employed had no influence on specific growth rate. It was clear that the SH containing amino acids as well as the vitamins not containing an SH group and inorganic reducing agents in themselves did not or only slightly increased the formation of OSL. By themselves neither cysteine nor ascorbic acid were effective, although their mixture was markedly increasing the formation of OSL by the strain H46A, while the other four strain combinations had little or no effect. Ascorbic acid is able to suspend the inhibitory effect of cystine, perhaps by driving the reversible oxidation—reduction of cystine—cysteine in the direction of the reduced form. This would support the view that in the course of growth or energy release an intensive electron release and uptake (oxidation—reduction) occurs, and on a culture medium having a low oxidative—reductive capacity, OSL formation may be limited. The results by MICKELSON [6] were indicative of a role of SH-enzymes in the energy release of streptococci, so he assumed glycerol-aldehyde-3-P-dehydrogenase to represent the point of inhibition. According to KRIMSKY and RACKER [5] glutathione is the prosthetic group of this enzyme. MICKELSON actually succeeded in suspending the inhibitory effect by glutathione and cysteine. These results make it probable that in the case of our strain the reducing agents exerted a similar effect. It seems a contradiction that we could not attain similar results with glutathione as with cysteine. We have to

suppose that in the case of cysteine, independently from its reducing effect, the specific chemical structure of the molecule has some importance in the mechanism of OSL formation. The fact that streptococci are transforming about 90% of the glucose into lactic acid [12], emphasizes the importance of lactic acid dehydrogenase.

We could establish that under fully identical conditions the two group C strains attained a higher cell count than the three group A strains. This difference in growth indicates that the serological groups differ not only in their group antigen but also in their metabolism. On the other hand, from the point of view of OSL formation, all the five strains behaved individually and failed to display any group-specific characteristics.

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EFFECT OF ENDOTOXIN ON THE SERUM LEVEL OF COMPLEMENT COMPONENTS

By

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(Received March 23, 1967)

Summary. The effect of *S. typhi* endotoxin on the serum level of complement and its components has been examined in rabbits. The titre of total complement and components C₂ and C₄ rose twofold 48 hours after an endotoxin injection; the level of the other two components was unaffected. None of the components showed a titre increase in endotoxin tolerant animals.

SPINK and VICK [1] described in 1961 the role in endotoxin shock of a thermolabile serum factor probably identical with complement. Later, several authors demonstrated that intravenous administration of endotoxin induced first a decrease then an increase in the complement level [2—6]. These investigations failed to give an answer as to which complement factors were involved and whether or not endotoxin tolerance influenced these changes. The present paper gives an account of investigations into this problem. For technical reasons the titres of the 4 classical complement factors were only determined.

Materials and methods

Endotoxin was prepared from *S. typhi* by the BOIVIN—MESROBEANU method.

Complement reagents. Reagents for the determination of the 4 classical complement factors were prepared from guinea pig serum by standard methods [7]. R1 and R2 were prepared by dialysis; for titration, these reagents were always supplemented with serum inactivated for 30 minutes (H). The reagents were used at the following dilutions and amounts: R1, 1 : 9, 0.30 ml; R2, 1 : 18, 0.15 ml; R3, R4 and H, 1 : 5, 0.30 ml. The reagents were standardized by determining the titres of components in guinea pig serum.

Sensitized sheep erythrocytes. A 2% suspension was prepared from sheep erythrocytes which had previously been washed three times and stored in the refrigerator. In one experiment suspensions originating from the same washing procedure were used. Each suspension was adjusted to a standard density by photometric measurement. Lyophilized haemolysin was diluted 1 : 2000.

Diluent. Saline containing 0.1% MgSO₄.

Titration of complement and its components. Blood samples taken from the marginal ear vein of rabbits were left to stand at room temperature for 1/2 hour then the clot was separated from the tube wall. After another 1/2 hour at room temperature and a subsequent 1/2 hour refrigeration, the samples were centrifuged. The sera were stored in the deep freeze at -16 to -18°C. Titration was carried out next day. Graded doses, each increasing by 0.05 ml, of the appropriate serum dilutions were pipetted into Wassermann tubes. Then the reagents were added and the volume in each tube was made up with the diluent to 2 ml. To each tube 1 ml sensitized sheep erythrocyte suspension was added. Incubation was done for 90 minutes at 37°C. Then the tubes were centrifuged and the degree of haemolysis was determined photometrically. CH₅₀ values were calculated from KABAT and MAYER's table [7].

Results

In preliminary experiments the relationship between time and change in total complement level was examined in 3 rabbits. The complement titre reached its peak 48 hours after the endotoxin injection and fell to its normal value in 10 to 14 days. As 1, 2 or 3 hours after the endotoxin injection there was no decrease in complement titre, in subsequent experiments 30 minute values were considered.

The animals were divided into 6 groups:

1. Five normal, untreated rabbits were injected intravenously with saline corresponding in amount to the applied dose of endotoxin. Blood samples of 4 to 6 ml were taken before and 30 minutes after the injection. Results are shown in Table I.

Table I
Effect of bleeding on the serum level of complement and complement components
Number of rabbits examined, 5

	T		C'1		C'2		C'3		C'4	
	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%
Initial value	29.1	100	272	100	38.3	100	200	100	78.7	100
30 minute value	28.8	99	272	100	37.4	97	208	104	82.6	105
Change	-0.3	-1	0	0	-0.9	-3	+8	+4	+3.9	+5

T = total complement level

CH₅₀ = 50% haemolytic unit/ml.

2. A second control group consisting of 5 rabbits received similar treatment except that the second sampling was performed 48 hours after the injection (Table II).

Table II
Effect of bleeding on the serum level of complement and complement components
Number of rabbits examined, 5

	T		C'1		C'2		C'3		C'4	
	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%
Initial value	25.7	100	246	100	29.4	100	254	100	58.6	100
48 hour value	25.2	98	251	102	30.2	103	255	100	65.3	112
Change	-0.5	-2	+5	+2	+0.8	+3	+1	0	+6.7	+12

T = total complement level

CH₅₀ = 50% haemolytic unit/ml

From Tables I and II it is seen that neither blood sampling nor the injection caused significant changes in complement and complement component titres.

3. The third group, consisting of 6 rabbits, received immediately after sampling $1/2$ LD₅₀ *S. typhi* endotoxin. The second sampling was performed 30 minutes later.

Table III

Effect of endotoxin on the serum level of complement and complement components
Number of rabbits examined, 6

	T		C'1		C'2		C'3		C'4	
	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%
Initial value	25.6	100	247	100	35.6	100	231	100	68.7	100
30 minute value	25.5	100	262	106	34.0	96	224	97	69.1	101
Change	-0.1	0	+15	+6	-1.6	-4	-7	-3	+0.4	+1

T = total complement level

CH₅₀ = 50% haemolytic unit/ml

From Table III it is evident that there was no change in the titre of complement and its components.

4. Six rabbits were sampled 48 hours after the injection of $1/2$ LD₅₀ *S. typhi* endotoxin.

Table IV

Effect of endotoxin on the serum level of complement and complement components
Number of rabbits examined, 6

	T		C'1		C'2		C'3		C'4	
	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%
Initial value	19.4	100	286	100	27.6	100	234	100	47.6	100
48 hour value	48.5	250	295	103	62.5	226	251	107	139.0	292
Change	+29.1	+150	+9	+3	+34.9	+126	+17	+7	+91.4	+192

T = total complement level

CH₅₀ = 50% haemolytic unit/ml

Table IV indicates that a considerable increase occurred in total complement level. As to the components, especially C'2 and C'4 showed increased titres. It should be noted that the highest rise was observed in the very animal showing the lowest initial titre.

5. Three animals received daily doses of $1/2$ LD₅₀ endotoxin for 6 days, then after a 48 hour interval another $1/2$ LD₅₀ dose. Blood samples taken 30 minutes after the last injection showed no significant change (Table V).

Table V

Effect of endotoxin on the serum level of complement and complement components in tolerant rabbits
Number of rabbits examined, 3

	T		C'1		C'2		C'3		C'4	
	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%
Initial value	25.3	100	229	100	38.8	100	194	100	87	100
30 minute value	25.0	99	235	102	37.4	96	195	100	89	102
Change	-0.3	-1	+6	+2	-1.4	-4	+1	0	+2	+2

T = total complement level

CH₅₀ = 50% haemolytic unit/ml

6. This group consisted of 5 endotoxin tolerant rabbits. Complement and component titres were examined 48 hours after one endotoxin injection (Table VI).

Table VI

Effect of endotoxin on the serum level of complement and complement components in tolerant rabbits
Number of rabbits examined, 5

	T		C'1		C'2		C'3		C'4	
	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%	CH ₅₀	%
Initial value	21.8	100	192	100	41.9	100	234	100	83.6	100
48 hour value	21.2	97	207	108	40.2	96	249	106	88.2	105
Change	-0.6	-3	+15	+8	-1.7	-4	+15	+6	+4.6	+5

T = total complement level

CH₅₀ = 50% haemolytic unit/ml

No significant changes were observed; the highest titres were: T, 119%; C'2, 119%; C'4, 132%. In 3 animals the titres decreased slightly.

Discussion

The role of complement in endotoxin shock was first assumed by SPINK and VICK [1]. Subsequently, SPINK [6] demonstrated that the decrease in complement titre was due to the fact that endotoxin induced an anaphylactic reaction which used up the complement. KOSTKA and STERZL [8] explained the phenomenon with the fixation of complement in the reaction between endotoxin and natural antibodies, while other authors [9] assumed some unknown mechanism. It seems obvious that endotoxin exerts an indirect effect, as doses inducing a considerable decrease in the complement level *in vivo* are ineffective *in vitro* [3]. The increase in complement level may be due to a rebound effect. KOHLER and SPINK [10] investigated the effect of endotoxin in dogs decomplicated with aggregated human gamma globulin; this method, however, also failed to elucidate the role of complement.

As to the total complement level, our results are not in complete agreement with data in the literature [2—5], as we have failed to demonstrate an initial decrease. This may indicate that rise is not due to a rebound effect but might be attributed to the direct action of endotoxin on complement-producing cells. This consideration is supported by the findings of THORBECKE *et al.* [11] and WILLIAMS *et al.* [12] *viz.* that the production *in vitro* of the two complement factors β_{1C} -globulin (a subcomponent of C'3) and β_{1E} -globulin (C'4) considerably increased in monkey and mouse liver tissue cultures if the donor animals had been treated with endotoxin 24 hours before the removal of their liver.

Our examinations clearly indicate that 48 hours after the endotoxin injection the titre of C'2 and C'4 significantly increases. However, an effective dose of endotoxin given to tolerant animals caused no rise either in total complement or in component titres. This finding was the more interesting as tolerant animals are resistant to different infections. The role of complement in the endotoxin-induced increase of non-specific resistance is, therefore, not quite clear.

The method used in the present study for the determination of complement component titres may not be the best for the purpose. It was elaborated by HEGEDŰS and GREINER [13] and modified by several authors [7]. In recent years this technique has been criticized by some investigators. Although the method has several disadvantages, it is still adequate, as emphasized by WEDGEWOOD [14], for obtaining significant biological information provided that the given criteria are applied rigorously.

Acknowledgement. The authors are indebted to DR. K. UJHELYI, Chief, Department of Vaccine Research, National Institute of Public Health, for the preparation of endotoxin.

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ANTIBODY PRODUCTION IN THE FROG*

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(Received April 11, 1967)

Summary. Frogs (*Rana esculenta*) kept at 8°C and 22°C were immunized with corpuscular and soluble antigens. *Salmonella typhi* was more effective than soluble antigens. Antibody production was more favourable at 22°C than at 8°C. Incomplete Freund adjuvant failed to increase antibody production in 4 weeks.

Antibodies were present mainly in the γ -globulin fraction. Ultracentrifugal analysis indicated that sera of the immunized animals contained components characterized by sedimentation coefficients 16, 6.8 and 5.

Investigations into the phylogenesis of immune response have shown that in invertebrates a phagocytic mechanism is primarily responsible for immunity [1—3]. Higher vertebrates show adaptive immunity characterized by specific antibody production, rejection of transplanted skin or other tissue, development of delayed allergy after antigen-adjuvant administration, and proliferation of mononuclear cells. The lowest animal shown to possess this complex reactivity is the lamprey (*Petromyzon marinus*) [4, 5].

Apart from some basic considerations, data are scarce concerning immunity in cold-blooded vertebrates. There are several contradictory findings even in field examined in some details such as the relationship between temperature and antibody production [6—10]. It seemed therefore interesting to examine immunity in certain cold-blooded vertebrates. The present paper gives an account of the immune response to corpuscular and soluble antigens in frogs kept at different temperatures. The immunological and physico-chemical properties of the produced antibodies have also been analysed.

Materials and methods

Animal. *Rana esculenta* was used.

Antigens. *S. typhi* vaccine containing 2×10^9 cells per ml served as a corpuscular antigen. Ovalbumin and bovine γ -globulin were used as soluble antigens. Ovalbumin was prepared as described by COLE [11] and recrystallized three times. Bovine γ -globulin was prepared according to KENDALL [12]. The soluble antigens were dissolved in 0.65% NaCl solution.

Immunization. The antigens were injected into the dorsal lymphatic sac. Antigens given with adjuvant were administered intramuscularly.

Group I of the animals received five 0.5 ml doses of *S. typhi* vaccine at three day intervals.

* Paper presented at the 9th International Congress of Microbiology, Moscow, July 1966.

Group II was immunized at three day intervals with a mixture of ovalbumin and γ -globulin. A total dose of 6.5 mg of each protein was injected in 15 days.

Group III served for the examination of the adjuvant effect; three 10 mg doses of bovine γ -globulin+incomplete Freund adjuvant were given intramuscularly at five day intervals. Twenty control frogs in this group received γ -globulin without adjuvant.

Animals in groups I and II were bled 7 days after the last injection. The serum antibody content in group III animals was examined on the 7th and 14th day after the last injection.

Antibody titration. *S. typhi* antibodies were determined by the tube agglutination technique using 0.65% NaCl as a diluent. The sera of frogs immunized with the soluble antigens were inactivated and examined by the plate agar diffusion [13] and tube precipitation techniques. Because of the small amount of individual sera no precise quantitative titration was performed.

Examination of serum proteins. Gel filtration analysis of serum proteins in untreated and immunized frogs was carried out as described by FLODIN and KILLANDER [14]. One ml of serum was run on 50×1 cm Sephadex G-200 column (pH=8; TRIS buffer, I = 0.1 μ , 20 ml/hour). Optical density of the fractions was measured with the Unicam SP spectrophotometer at 280 m μ . Antibody content of "macroglobulin" (I) and "7S" (II) fractions was determined after concentration by agar diffusion and tube precipitation in the case of soluble antigens, and by agglutination in the case of *S. typhi*.

Immune electrophoresis of frog serum was performed with rabbit anti-frog serum by the micromethod of SCHEIDEGGER [15] using MICHAELIS buffer (pH=8.2; I=0.1 μ). Precipitation lines were recorded either by direct photographs or by staining with Amidoschwarz 10 B after elution and drying.

Results

A total of 100 frogs were immunized with *S. typhi*. Animals in group Ia were kept at 20 to 22°C throughout immunization. Table I shows that the serum of 56 out of 60 animals gave agglutination, the maximum titre being 1:1280. Members of group Ib (40 frogs) were kept at a temperature of 6 to 8°C, until the 18th day when the group was divided into two subgroups; 20 animals were left at 6 to 8°C for a further period of 3 days, the remaining 20 frogs were kept for 72 hours at 20 to 22°C. In group Ib no significant titres were demonstrated for *S. typhi* (Table I).

Table I

Agglutination titre in frogs immunized at different ambient temperatures with S. typhi vaccine

Group of animals	Titre of 0 agglutination									Control
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
Ia Immunized at 18–22°C	56/60*	56/60	56/60	32/60	30/60	27/60	18/60	9/60	0/60	0/10
Ib Immunized at 6–8°C	0/40	0/40	0/40	0/40	0/40	0/40	0/40	0/40	0/40	0/10
Immunized at 6–8°C then kept at 24°C for 72 hours	3/20	2/20	2/20	0/20	0/20	0/20	0/20	0/20	0/20	0/10

* Numerator: number of animals showing the corresponding titres; denominator: number of animals immunized.

The localization of *S. typhi* antibodies was determined by antibody analysis of gel filtration fractions of the immune sera. The antibodies were shown mainly in the "macroglobulin" (I) and less in the "7S" fraction (II) (Fig. 1).

Results obtained in group II animals, which had been immunized simultaneously with the two soluble antigens, indicated that every frog produced antibodies against bovine γ -globulin. Antibodies reacting with ovalbumin were,

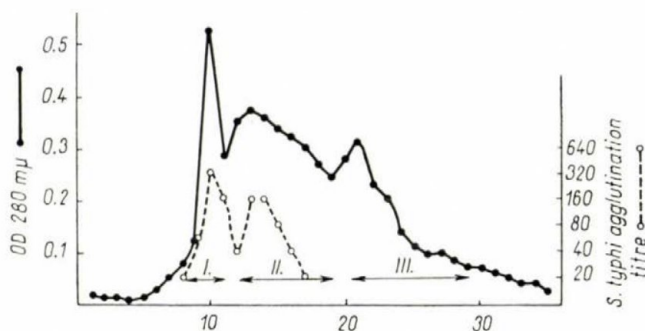


Fig. 1. Gel filtration examination of serum of frogs immunized with *S. typhi*. Sephadex G-200. I = "macroglobulin" fraction, II = "7S" fraction

however, demonstrated only in 12 out of the 20 frogs. In group III 5 animals out of 20 died during immunization with adjuvant + γ -globulin. All control animals which received only bovine γ -globulin survived. The results are shown in Table II.

Table II

Antibody production in frogs immunized with soluble antigens

Antigens	Antibody production	Precipitation titre*	
		Bovine γ -globulin μ gN/ml	Ovalbumin μ gN/ml
Bovine γ -globulin 6.5 mg	20/20**		
+			
Ovalbumin 6.5 mg	12/20	70	150
Bovine γ -globulin 10 mg			
+	15/20	300	—
Incomplete Freund adjuvant			
Bovine γ -globulin 10 mg	20/20	18	—

*Antigen precipitated by 0.5 ml undiluted frog immune serum

**Numerator: number of antibody producing animals; denominator: number of immunized animals

After gel filtration of sera prepared in frogs with bovine γ -globulin and adjuvant + bovine γ -globulin the "macroglobulin" and "7S" fractions were concentrated and examined for antibody titre. Definite precipitation was observed in both sera only with fraction "7S".

The serum spectrum of untreated and bovine γ -globulin-treated frogs was compared in immunoelectrophoretic experiments. Serum samples taken from frogs immunized in summer and in autumn contained 12 to 14 compo-

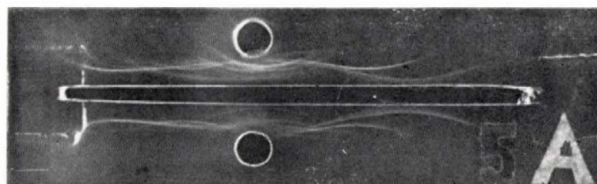


Fig. 2. Immunoelectrophoresis of control (5A) and immunized (unmarked) frog sera

nents. The complexity of the γ -globulin area in immunized frogs was noticeable: it consisted of components corresponding in mobility to IgA, IgM and IgG fractions (Fig. 2).

Ultracentrifugal analysis of the sera of untreated and of bovine γ -globulin-treated frogs showed that the control sera contained components characterized by sedimentation coefficients 17–18, 8.0–8.5 and 4.0–4.8; in the serum of immunized animals the components were characterized by coefficients 15–16, 6.5–6.8 and 4.6–5.0.

Discussion

In the frog immunized with *S. typhi* the production and distribution of antibodies was found to depend considerably on the environmental temperature. According to TRNKA and FRANEK [16] the most important factor is the amount of antigen, as suitable doses induce antibody production even at low temperatures. ELEK, REES and GOWING [17] confirming the finding of BISSET [9] showed that antibody production took place at low temperatures in *Xenopus laevis*, but the antibodies entered the circulation only when ambient temperature was raised to 27°C for 60 hours. According to EVANS [10] and MAUNG [18] there was no antibody production at low temperatures.

In our experiments *S. typhi* antigen was given in high doses and part of the animals immunized at 8°C was subsequently kept at 22°C for at least 72 hours. The results have shown ambient temperature to be an important factor in antibody production. This seems to confirm the opinion of MAUNG [18] and

ALCOCK [19] that definite antibody production commences only at higher temperatures.

Of the three kinds of antigens the corpuscular *S. typhi* vaccine was the best immunizing agent. Ovalbumin induced the weakest antibody production. BARNETT, SOUDA and SANFORD [20] demonstrated in rats that thymectomy exerted no appreciable effect on antibody production against *E. coli* but inhibited the development of immunity against BSA. It was found by PINNAS and FITCH [21] that in thymectomized rats *S. typhi* was more antigenic than bovine serum albumin. These findings indicate some similarity between the immune response of cold-blooded and thymectomized warm-blooded animals.

In our experiments the incomplete Freund adjuvant was toxic and, at least in 30 days' observation, did not enhance antibody production. AMBROSIUS and LEHMANN [22] described that the incomplete Freund adjuvant was successfully applied in tortoises.

Physicochemical examinations showed that frog serum contained considerably less protein components than the serum of mammals. This finding agrees with recent observations of MARCHALONIS and EDELMAN [23]. It is interesting and important that frog serum antibodies are present mainly in the γ -globulin fraction. Antibodies produced against the corpuscular antigen appear in the "macroglobulin" and less in the "7S" fraction, while the majority of those induced by soluble antigens are present in "7S". Ultracentrifugal analysis also indicated a similarity between the serum components of the frog and higher animals.

Acknowledgement. The authors are indebted to Mr. M. SZABOLCS, Central Laboratory, University Medical School, Debrecen, for performing the ultracentrifugal analyses.

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THE ANTIGEN-FIXING CAPACITY OF TISSUES OF SENSITIZED GUINEA PIGS

By

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(Received April 14, 1967)

Summary. Each gram of the lung and heart tissues of actively sensitized guinea pigs killed during anaphylactic shock has been found to fix 0.5 to 1.5 μgN ^{51}Cr -labelled ovalbumin antigen immunospecifically. A method has been evolved for estimating the antigen-fixing capacity of the tissues of actively sensitized guinea pigs. After passive sensitization such a correlation could not be demonstrated *in vitro* since homogenization and centrifugation separate the antibody from the tissues. The results pointed to the probable role of the heart besides that of the lung in the pathomechanism of anaphylactic shock in the guinea pig.

Since DALE's [1] classical experiments anaphylactic phenomena have generally been attributed to the reaction with the homologous antigen of tissue-fixed (tissular, cellular, sessile or cytophile) antibodies. Their importance was further supported by the recognition of their decisive role in tuberculin-type hypersensitivity and in transplantation immunity. Several methods have been developed for the demonstration of tissue antibodies; REISS *et al.* [2] described agglutinating antibodies in lymph node cells; HAYES *et al.* [3] applied tissue and cell films; tissue antigens were localized by histochemical and fluorescence technique [4] as well as by autoradiography [5].

The use of radioisotopes seemed to be promising in two ways, *viz.* (i) quantitative determination of the injected labelled antigen taken up *in vivo* by the tissues of immunized animals [6] or *in vitro* by spleen and lymph node cells of sensitized animals [7]; (ii) demonstration of the fixation of labelled antibodies to cells *in vivo* and *in vitro* [8, 9, 10].

In this Institute a new radioisotope method for the quantitative determination of tissue antibodies based on LANDSTEINER and HALBAN's haptene inhibition principle was evolved. Besides, a microquantitative assay based on the antienzymatic method has been described [11, 12].

In the present work the antigen-fixing capacity of guinea pig tissues was studied with labelled antigens in order to estimate the quantity and role of tissue antibodies in anaphylactic shock. The applied method was based on our observation that the non-specific adsorption to homogenized tissues can be reduced to an approximately constant level under appropriate conditions [13].

Materials and methods

Antigens. Three times recrystallized ovalbumin (OA), ^{51}Cr -labelled chromium ovalbumin (^{51}Cr -OA) [14] and ^{131}I -labelled iodo-ovalbumin (^{131}I -OA) [15] were prepared in this Institute. The labelled proteins were gel-filtered on Sephadex G-200 columns and the pure OA fraction was used.

Antibody. The antibody in the sera of rabbits immunized with OA was precipitated with the homologous antigen, isolated on Sephadex G-200 gel [16] and labelled with ^{131}I [15].

Sensitization and shock. Male and female guinea pigs of 300 to 400 g weight were used. Different groups of animals were sensitized actively or passively. Active sensitization was carried out by subcutaneous injection of 2×4 mg of inactive Cr-OA, passive sensitization by injecting 0.48 mg per kg body weight of rabbit anti-ovalbumin-N intracardially. Anaphylactic shock was challenged 21 or 22 days after active sensitization, and 48 hours after passive sensitization, by injecting 4 mg of ^{51}Cr -OA into the jugular vein. Non-sensitized control animals were given the same injection and exsanguinated five minutes later.

Preparation of organs for the determination of antigen fixation. In experiments *in vivo* the sensitized animals were exsanguinated after challenge immediately before death. Their heart, lungs, liver and kidneys were removed and perfused with saline through the veins. The wet organs were weighed, minced and 1 g of each organ was placed in a centrifuge tube. The tubes were cooled to $+4^\circ\text{C}$ and the minced organs were washed three times with cold saline of pH 7.0 as usual for quantitative precipitation tests. The radioactivity of each supernatant, washing fluid and pulp was measured. Activity in the third washing was negligible.

In experiments *in vitro*, guinea pigs were exsanguinated 21–22 days and 48 hours after active and passive sensitization, respectively, and 1 g of each organ was homogenized with 2 ml saline of pH 7.0 in a Potter homogenizer to reveal all antibodies present in the organ. The homogenates were inactivated at 56°C .

The pH of the organ homogenates whose protein-fixing capacity was to be determined was adjusted to 6.9–7.1 with tris buffer (hydroxymethyl amino-methane). Then 6.7, 10, 13 or $19.5 \mu\text{g N/ml}$ ^{51}Cr -OA was added to each homogenate. The mixtures were incubated at 37°C , then cooled to $+4^\circ\text{C}$ and centrifuged at 10 000 r. p. m. for 20 minutes. Subsequently, the precipitates were washed three times with cold saline.

Haemoglobin assay. Since washing of the organs until the total removal of blood would reduce their tissue antibody content, haemoglobin was determined in the homogenates by a method described earlier [13] and the antigen-activity in organ's blood was subtracted from the total.

Measuring of activity. Activity in the washed homogenates was measured by a well-type scintillation counter containing NaI (Tl) Bohrloch crystal, and calculated for N by comparison to a ^{51}Cr -OA standard of known N content. The Figures show the N contents calculated from the activities. The results were evaluated statistically.

Results

First, the effect of increasing antigen doses on non-specific antigen-fixation by lung and liver tissue was estimated *in vitro* (Fig. 1).

The organs of sensitized and non-sensitized guinea pigs fixed increasing amount of ^{51}Cr -OA when the amount of the added antigen was increased. After subtraction of the control values from those for antigen-fixation to the organs of sensitized animals, the corrected fixation value was approximately the same for each of the three antigen doses, indicating that specific fixation was hardly influenced by raising of the antigen dose; an antigen dose of $10 \mu\text{gN}$ was therefore sufficient to fix the antibody which had not been removed from the homogenate by washing.

Next, antigen fixation to lung, heart, liver and kidney tissue of actively or passively sensitized guinea pigs was determined *in vitro* and compared to the control values (Fig. 2).

Each organ of the actively sensitized animals fixed more antigen than the control one. In the passively sensitized group, on the other hand, activity in

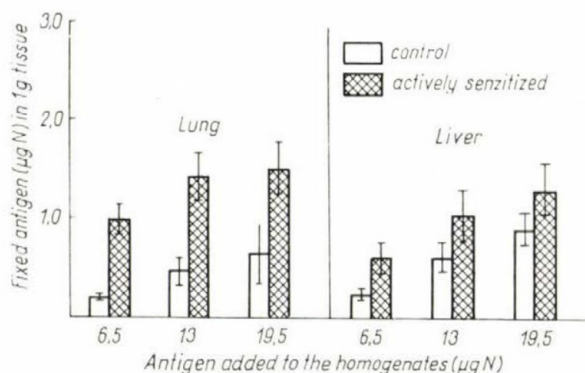


Fig. 1. Antigen fixation *in vitro* by organs of sensitized guinea pigs in the function of antigen concentration. Control: 5 animals; actively sensitized: 5 animals

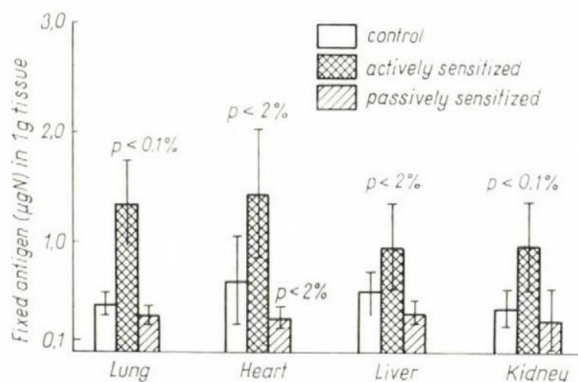


Fig. 2. Antigen fixation *in vitro* by organs of sensitized guinea pigs. Control: 10 animals; actively sensitized: 10 animals; passively sensitized: 10 animals

each of the organs was indicative of a decreased antigen fixation. The difference for the liver and the heart was significant, indicating that from the organs of passively sensitized animals not only the antigen but also the antibody had been washed off in the course of preparation.

To elucidate this question, animals were sensitized passively with five-fold doses, i.e., 2.4 mg N per kg body weight of antibody and 10 µg N antigen were added to each of the homogenates of the organs removed 48 hours later;

the antigen-antibody reaction proceeded under *in vitro* conditions. Considering the weak energy spectrum of ^{51}Cr , we performed parallel experiments with ^{131}I (Fig. 3).

The results of the two series of experiments were consistent with those presented in Fig. 2, indicating that, as tested *in vitro*, in animals sensitized with a large dose of antibody labelled either with ^{131}I (upper columns) or ^{51}Cr (lower columns) the amount of tissue-fixed antigen failed to rise over the control level.

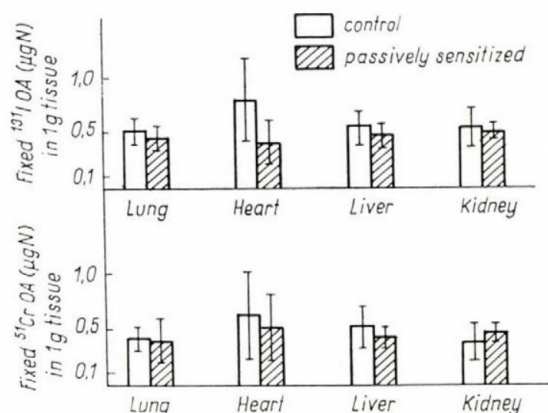


Fig. 3. Antigen fixation *in vitro* by organs of guinea pigs sensitized with 2.4 mg antibody N per kg body weight. Antigen: ^{131}I -OA (upper columns) and ^{51}Cr -OA (lower columns). Control: 5 and 10 animals, respectively; passively sensitized: 5 and 5 animals, respectively

However, even these data provided only indirect evidence of having washed off antibodies from the organs. For this reason, rabbit anti-OA was isolated on Sephadex G-200 gel [16] and labelled with ^{131}I . Three guinea pigs were sensitized passively with the ^{131}I -anti-OA (0.48 mg N per kg body weight). 48 hours later the animals were exsanguinated and OA antigen was added to their organ homogenates. The losses of antibody that had occurred in the course of preparation were estimated (Table I).

The supernatants obtained by centrifuging the homogenates contained a substantial part of the antibody activity and in the homogenates there remained hardly any activity. We have therefore assumed that in animals sensitized passively with heterologous immunoglobulins the fixation of antibody to tissues is due to weak physico-chemical bonds which fail to resist homogenization; consequently, a substantial part of the antibodies is removable with the supernatant after centrifugation. The antibodies produced by the actively sensitized animals, on the other hand, form more stable bonds with the tissues.

Table I

Antibody content in organs of guinea pigs sensitized with 0.48 mg per kg body weight of ^{131}I -labelled rabbit ovalbumin and the loss of antibody in the course of preparation

Guinea pig No.	Organ	Antibody in $\mu\text{g N}$				
		total in 1 g organ	in the supernatant of the centrifugate	in 1st washing fluid	in 2nd washing fluid	in residual tissue homogenate
1.	liver	0.33	0.20	0.04	0.02	0.07
	lung	0.96	0.69	0.09	0.02	0.16
	heart	0.48	0.33	0.05	0.04	0.06
	intestine	0.22	0.14	0.02	0.01	0.05
2.	liver	0.29	0.20	0.02	0.01	0.06
	lung	0.91	0.63	0.13	0.03	0.12
	heart	0.55	0.35	0.05	0.02	0.13
	intestine	0.05	0.09	0.03	0.03	0.02
3.	liver	0.46	0.29	0.05	0.03	0.09
	lung	0.86	0.66	0.03	0.06	0.11
	heart	0.63	0.48	0.07	0.03	0.05
	intestine	0.16	0.11	0.02	0.00	0.03

In experiments *in vivo* first of all the degree of elimination of antigen from the blood during anaphylactic shock was estimated in both actively and passively sensitized guinea pigs. The control animals were exsanguinated 5 minutes after the injection of the antigen into the jugular vein, the sensitized animals during shock, immediately before death.

Fig. 4 shows that in the blood of animals killed during anaphylactic shock the antigen level, as indicated by the activity values, was substantially decreased. Elimination of the antigen from the blood, presumably being the function of the sessile antibody content of tissues, is a good indicator of the degree of sensitization. In the blood samples from the guinea pigs sensitized actively and passively, the concentration of antigen was reduced by 45% and 34%, respectively, as compared to the control values.

In further experiments *in vivo*, antigen fixation by the organs of passively and actively sensitized guinea pigs was estimated during anaphylactic shock. Sensitized animals were given 4 mg ^{51}Cr -OA into the jugular vein and their organs were removed for the estimation of antigen fixation immediately before death.

In accordance with KESZTYŰS *et al.* [17], there was a substantial antigen excess in the lungs and the heart in both sensitized groups, but no appreciable excess in the liver and in the kidneys (Fig. 5).

The results obtained *in vivo* agreed well with the antigen accumulation

observed *in vitro* in relation to the lungs and the heart, but not in relation to liver and kidneys. Furthermore, in relation to the heart and lungs, antigen activity was significantly elevated in the passively sensitized animals.

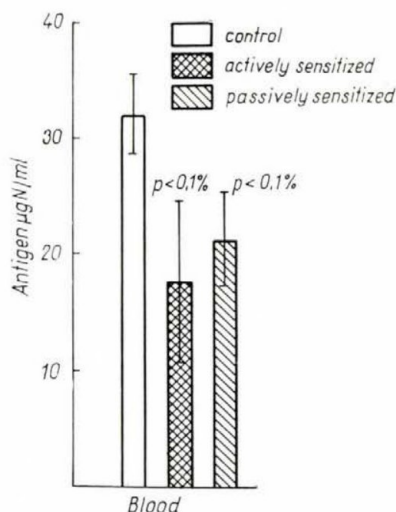


Fig. 4. Antigen concentration in the blood of sensitized guinea pigs during anaphylactic shock. Control: 8 animals; actively sensitized: 9 animals; passively sensitized: 10 animals

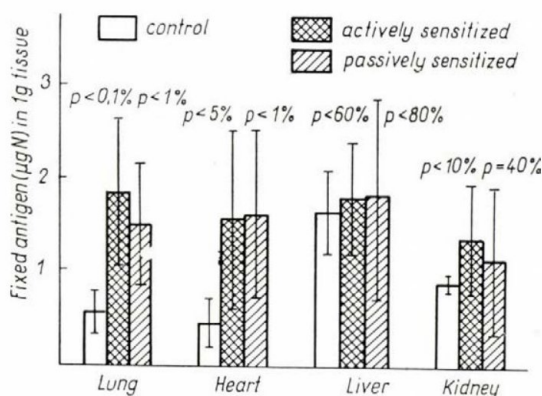


Fig. 5. Antigen fixation by different organs of sensitized guinea pigs during anaphylactic shock. Control: 8 animals; actively sensitized: 9 animals; passively sensitized: 10 animals

It should, however, be noted, that in these experiments the tissues had not been homogenized but minced. Consequently, one must be careful in drawing conclusions from antigen fixation *in vitro* for the phenomena taking place in the living animal.

Discussion

Our attempts at determining the antigen-fixing capacity of the guinea pig tissues have revealed that in the lung and heart of the animals a great amount of antigen had accumulated in the course of anaphylactic shock, most probably as a result of the antigen-antibody reaction. According to the consistent results of experiments *in vitro* and *in vivo*, neutralization of the antibody present in 1 g of heart and lung tissue needed 0.5–1.5 μ g N of antigen. However, the results of our experiments *in vivo* would have been more convincing had appropriate control values been available. During anaphylactic shock numerous factors (circulation, pH, anoxia, pulmonary air content, etc.) obviously undergo a change each of which may significantly affect the antigen fixation. Consequently, one should be cautious in estimating the quantity of sessile antibodies on the basis of the increased or decreased antigen fixation in different organs. According to the present results the antigen-fixing capacity of the lung, the shock organ of the guinea pig, is substantially greater in sensitized than in control animals. Besides, the role of the heart should be considered in the pathomechanism of the guinea pig's anaphylaxis.

When the antigen had been added to the organ homogenates *in vitro*, a considerably increased antigen fixation took place in the liver and kidney tissues of actively sensitized guinea pigs, but not in those of passively sensitized ones. Furthermore, in the course of anaphylaxis *in vivo*, the amount of fixed antigen increased neither in the liver nor in the kidneys. KESZTYŰS *et al.* [11] detected immuno-specifically fixed antigen in the kidneys and liver of immunized rabbits as well as of sensitized guinea pigs under conditions *in vitro*. We cannot offer any adequate explanation for our results since neither the liver nor the kidneys may be considered antibody-producing organs. It is nevertheless possible that antibodies are stored in certain cells of both organs, e.g., in their mast cells [18].

BRODER and SCHILD [19] injected soluble antigen-antibody complexes prepared in the zone of the antigen excess into the pulmonal artery of perfused, non-sensitized guinea pig lungs. Bronchial constriction was observed and in the perfusion fluid histamine and SRS (slow reacting substance) were detected. This phenomenon closely resembles the anaphylactic shock of the isolated lung. Considering that the biologically active mediators were liberated by a soluble antigen-antibody complex and that the amount of the challenging antigen was 50 to 100-fold of that necessary for the equivalency zone, the possible role in anaphylaxis *in vivo* of circulating antibodies cannot be excluded. In the quantitative estimation of tissue antibodies, especially if it is based on the activity of the labelled antigen, these considerations should not be neglected.

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THE MECHANISM OF ANTIBACTERIAL ACTION OF RAPHANIN

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(Received April 17, 1967)

Summary. Raphanin, the antibacterial substance of radish seed (*Raphanus sativus*) has been found to have a non-competitive inhibitory action on the SH enzymes urease and succino-dehydrogenase. It had no effect on enzymes lacking an SH group, such as alkaline phosphatase or glutamic acid—oxaloacetic acid transaminase. The mechanism of antibacterial action of raphanin may be due to its SH-enzyme inactivating effect.

IVÁNOVICS and HORVÁTH [1] isolated from radish seeds (*Raphanus sativus*) an oily substance having an antibacterial effect on both Gram negative and Gram positive bacteria; the substance was termed raphanin [2]. Raphanin was shown to have some additional activities; it inhibited the germination of seeds of various plants. The substance showed a notable toxicity, damaging fibroblast cultures at 1 : 20 000 dilution and being lethal for mice in single doses of 350 mg/kg. Thus its therapeutic application has been beyond consideration.

Later SCHMIDT and KARRER [3] have also described an antibacterial substance isolated from radish seed, termed sulphoraphene. The physical, chemical and biological characteristics of raphanin and sulphoraphene suggested the identity of the two substances. By isolating identical cleavage products from both substances, KOCZKA and IVÁNOVICS [4] verified the suspected identity.

The antibacterial effect of raphanin depends on its concentration. Low concentrations are bacteriostatic, higher ones bacteriocidal [5].

Various thiol compounds (cysteine, glutathione, thioglycolic acid, etc.) were found to inactivate raphanin [6]. Therefore, IVÁNOVICS supposed that raphanin exerts its antibacterial effect through combination with certain biologically important SH-groups [6]. Several antibiotics of different chemical composition and mechanism of action are known to be inactivated by thiol compounds. As typical examples let us refer to penicillin [7, 8] and streptomycin [9, 10]. To elucidate the mechanism of action of raphanin, it seemed desirable to examine whether the substance reacted specifically with some SH-containing enzymes. In these model experiments, crystalline urease and bacterial succino-dehydrogenase released by sonication were examined. For com-

parison's sake, two enzymes not containing SH were also studied, *viz.* crystalline alkaline phosphatase and glutamic acid—oxaloacetic acid transaminase obtained from bacteria lysed by toluene.

Materials and methods

Raphanin was obtained according to IVÁNOVICS and HORVÁTH [2].

E. coli B was cultured on a minimal medium containing in 1000 ml distilled water 3 g NaH_2PO_4 , 7 g K_2HPO_4 , 2 g NH_4Cl , 1 g NaCl, 0.6 ml FeCl_3 (0.01 M). After sterilization, 2 ml MgSO_4 (1.0 M) and 5 ml of 40% glucose solution were added and the pH was adjusted to 7.4.

Complete medium consisted of a minimal medium supplemented with 1% tryptone (Oxoid) and 0.5% yeast extract (Difco).

A stock solution of crystalline urease ($3 \times \text{N. F.}$, Nutritional Biochemicals Corporation, Cleveland, Ohio) of 300 m μ M concentration was prepared in pH 7.2, 0.2 M tris-HCl buffer containing 0.001 M versene.

Urease assay. To 1 ml enzyme solution was added 1 ml 0.1 M urea solution. The mixture was incubated in a 20°C water bath for 10 minutes. This was followed by the addition of 0.5 ml 20% NaOH, 7 ml distilled water and 0.5 ml NESSLER's reagent. The amount of ammonia produced was determined photometrically at 470 m μ wavelength in a Spectromom 201 (MOM, Budapest) apparatus.

Succino-dehydrogenase assay. This was performed as described by DOMJÁN and KOVÁCS [11]. As enzyme preparation, sonicated suspension of *E. coli B* was used. Bacterial suspension grown in complete medium and having an optical density of 1 (800 $\mu\text{g/ml}$ protein) was centrifuged at 6000 r.p.m. for 10 minutes and washed twice with cold 0.1 M pH 7.3 phosphate buffer. The sediment was resuspended in 20 ml buffer and sonicated for a 50-second period (MSE Ultrasonic Power Unit, England). To 1.5 ml of the suspension thus obtained, an equal amount of 0.02 M Na-succinate was added. Each tube was covered with 3 ml paraffin oil and after thorough stirring, the samples were transferred into a 27°C water bath, incubated for 10 minutes, and subsequently 0.05 ml of 0.2% methylene blue was pipetted into the watery phase of the system. Magnetic stirring was repeated to achieve uniform mixing. The time required for decolouration of the methylene blue was measured.

Alkaline phosphatase assay. Lyophilized enzyme of bovine origin (Reanal, Budapest) was dissolved at a concentration of 0.1% in 0.2 M tris buffer of pH 9.0. One ml of this solution was added to an equal amount of a 0.02 M aqueous disodium p-nitrophenyl-o-phosphate solution. The mixture was incubated for 10 minutes in a 37°C water bath, then transferred into an ice bath. To stop enzyme activity, to each tube 3 ml of a mixture of 0.05 M Na-carbonate and 0.1 M Na-pyrophosphate was added [12]. The released amount of paranitrophenol was measured photometrically at 420 m μ wavelength.

Glutamic acid—oxaloacetic acid transaminase assay. A suspension of *E. coli B* lysed by toluene was used as enzyme preparation. Bacteria were grown in 200 ml complete medium until an optical density of 0.5 had been reached, then the culture was centrifuged at 600 r.p.m. for 10 minutes, the sediment was washed twice with cold saline, the final sediment was resuspended in 20 ml saline, 0.5 ml toluene was added and the mixture was shaken for 2 minutes. The extract thus obtained was used as an enzyme preparation and its activity was measured as described by DUBACH [13].

In the experiments, raphanin was added to the enzymes at different concentrations and the mixtures were preincubated for 15 minutes at temperatures appropriate for the assay of the actual enzyme.

Results

The biological activity of the raphanin preparation was determined by serial dilution in minimal as well as in complete medium. To each dilution of raphanin 10⁶/ml *E. coli B* was added and the cultures were incubated for 24

hours. The fully inhibitory concentration in minimal and complete medium was 25 $\mu\text{g/ml}$ and more than 100 $\mu\text{g/ml}$, respectively. Analysis of the phenomenon has shown that this difference could be ascribed to the presence of casamino acid in the medium. Among the amino acids present, only cysteine exhibited an inhibitory action. This observation correlates well with the results of earlier studies [6].

The action of raphanin on urease was examined in the presence of 50, 100, 200, 250, 300, 400, and 500 $\mu\text{g/ml}$ of raphanin. The enzyme concentration in each tube was 300 $\text{m}\mu\text{M/ml}$. The mixtures were incubated at 30°C for 15

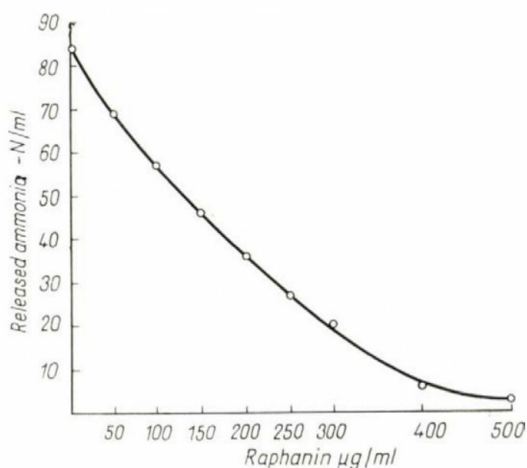


Fig. 1. Effect of raphanin on urease activity

minutes, then transferred into a water bath of 20°C. One ml of 0.1 *M* urea was added per tube and the amount of ammonia released after 10 minutes incubation was determined and expressed in terms of nitrogen equivalent. Results are shown in Fig. 1.

The raphanin concentration causing 50% inhibition of enzyme activity was estimated. Subsequently, enzyme activity was examined in the presence of 200 or 250 $\mu\text{g/ml}$ raphanin and various concentrations of the substrate within the range of 0.1–0.0005 *M*. Results are shown in Fig. 2.

Raphanin was found to inhibit urease non-competitively. In the next experiment, 25 or 100 $\mu\text{g/ml}$ of cysteine was added to a mixture of 300 $\text{m}\mu\text{M/ml}$ enzyme and 200 $\mu\text{g/ml}$ raphanin. The samples were incubated for 15 minutes at 30°C, subsequently transferred to a 20°C water bath and urease activity was estimated at substrate concentrations of 0.1 to 0.0005 *M*. The urease inactivating effect of 200 $\mu\text{g/ml}$ raphanin was practically completely inhibited by 100 $\mu\text{g/ml}$ of cysteine. Twenty-five $\mu\text{g/ml}$ of cysteine had a 50% effect. Results are presented in Fig. 3.

The effect of raphanin on succinodehydrogenase was also studied. Increasing concentration of raphanin caused increasing reduction of enzyme activity, *viz.* an increasing methylene blue decolouration rate. Results are presented in Table I.

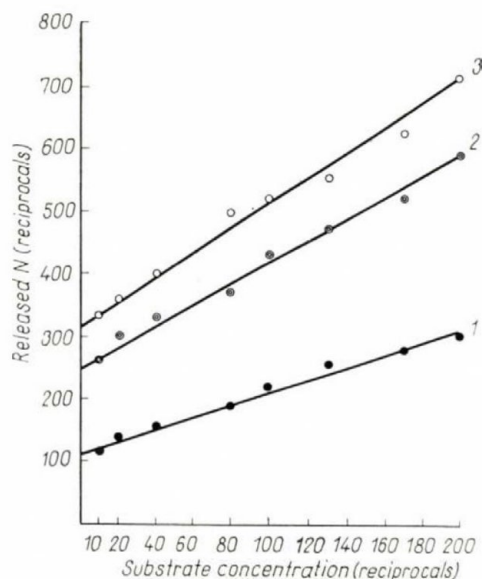


Fig. 2. Urease activity at various raphanin concentrations. 1 = Enzyme activity of control (not treated with raphanin), 2 = Enzyme activity at 200 $\mu\text{g/ml}$ of raphanin, 3 = Enzyme activity at 250 $\mu\text{g/ml}$ of raphanin

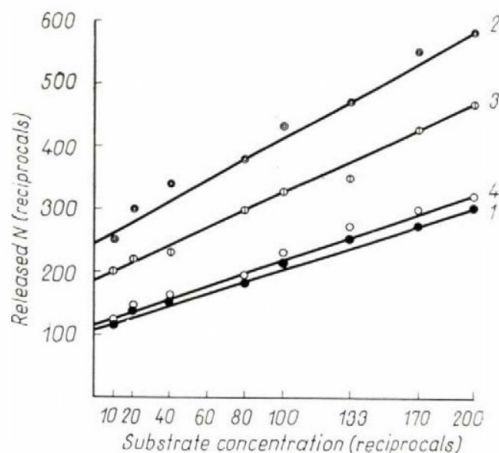


Fig. 3. Inhibition of raphanin effect with cysteine. 1 = Enzyme activity of control, 2 = Enzyme activity in the presence of 200 $\mu\text{g/ml}$ of raphanin, 3 = Enzyme activity in the presence of 200 $\mu\text{g/ml}$ of raphanin and 25 $\mu\text{g/ml}$ of cysteine, 4 = Enzyme activity in the presence of 200 $\mu\text{g/ml}$ of raphanin and 100 $\mu\text{g/ml}$ of cysteine

Table I

Succinodehydrogenase activity in the presence of raphanin at various concentrations

Raphanin concentration ($\mu\text{g/ml}$)	0	20	100	200	400	500
Decolouration time of methylene blue (minutes)	2	4	12	22	43	60

In further experiments the effect of raphanin on alkaline phosphatase and glutamic acid-oxaloacetic acid transaminase activity was examined. After addition of 100–5 000 $\mu\text{g/ml}$ of raphanin, the samples were incubated for 15 minutes at 37°C, then the appropriate substrates were added and incubation was continued at 37°C — with transaminase at room temperature — for further 10 minutes. Subsequently, enzyme activity was stopped and estimated. These experiments have shown that even 5 000 $\mu\text{g/ml}$ of raphanin failed to inhibit both alkaline phosphatase and glutamic acid-oxaloacetic acid transaminase activity.

Discussion

Studies of the influence of raphanin on the activity of SH- and non-SH-type enzymes have shown that the substance is not a general enzyme poison. It had no effect whatever on glutamic acid-oxaloacetic acid transaminase and alkaline phosphatase activities. The fact that it inhibited urease and succinodehydrogenase activity supports the assumption [6] that raphanin exerts its antibacterial action by combining with some important SH-groups. Raphanin inhibited urease activity in a non-competitive way. The antibacterial effect was explained partly by an inhibition of vital processes through suppressing the activity of SH-enzymes, partly by the observation [14] that enzyme inhibition brought about by SH-reagents at a concentration not yet toxic for cell respiration and movement is already inhibitory to cellular division.

Acknowledgements. The author is indebted to Prof. G. IVÁNOVICS and DR. L. ALFÖLDI for help and valuable criticism.

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THE EFFECT OF HAEMOCOAGULASE (REPTILASE) ON THE LOCAL SHWARTZMAN PHENOMENON

By

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(Received April 22, 1967)

Summary. Local Schwartzman phenomenon was induced in rabbits. Intravenous injection of haemocoagulase, 1 unit immediately before challenge and 1 unit two hours later definitely enhanced haemorrhage and necrosis. Haemocoagulase exerts no effect on complement activity in vitro.

Several authors have suggested that a clotting disturbance was a substantial, if not the exclusive factor in the pathomechanism of the local Schwartzman phenomenon. GOOD and THOMAS [1] and CLUFF and BERTHRONG [2] could prevent the phenomenon by heparin. RALL *et al.* [3] have shown that ethyl biscoumaracetate (Tromexan) was as effective an inhibitor as heparin; MCKAY and SHAPIRO [4], HOROWITZ *et al.* [5] and KLEINMAIER *et al.* [6] observed a significantly shortened coagulation time in rabbits after challenge with endotoxin.

It seemed therefore justified to study the effect on the local Shwarzman phenomenon of haemocoagulase, a haemostatic extensively applied in human therapy. Furthermore, we supposed that haemocoagulase would enhance the reaction and thus shed light from a new side on the role of blood coagulation in Schwartzman's phenomenon, especially as the question posed by HALPERN *et al.* [6] whether heparin acted on the Schwartzman phenomenon as an anti-coagulant or through its anticomplementary effect has not yet been answered unequivocally.

Materials and methods

The haemocoagulase (Reptilase, Disperga) was prepared from the venom of the snakes *Bothrops jararaca* and *Lachesis atrox*. The extract which has a thrombin and thrombokinase-like activity, was purified to eliminate neurotoxins and some ballast materials. One Klobusitzky unit of haemocoagulase coagulates 5 ml Ca-free horse serum at 20°C in 10 minutes.

Animals. As experimental animals rabbits were used, 10 in each experimental and control group.

Procedures. Schwartzman phenomenon was induced by the semiquantitative method evolved by KESZTYŰS *et al.* [8]. As preparing dose 0.25 mg *E. coli* 0 111 endotoxin and its serial twofold dilutions were administered intradermally in volumes of 0.4 ml. Control rabbits were given 0.4 ml saline. As a challenging dose 1 mg endotoxin was injected and the result was read 24 hours later. The cutaneous reaction was characterized by the size of the haemorrhagic area.

One Klobusitzky unit of haemocoagulase was administered intravenously immediately before, and 2 hours after, challenge.

In studying the effect of the drug on the complement, 0.2 and 0.02 unit of haemocoagulase was used in *in vitro* experiments.

Results and discussion

As seen in Fig. 1, the haemorrhagic area decreased with the rising dilution of endotoxin. The highest dilution (1 : 16) caused no reaction in the control animals.

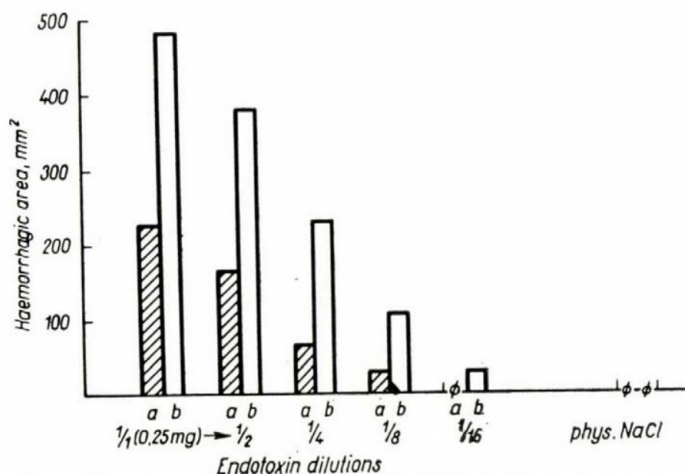


Fig. 1. The effect of haemocoagulase on local Schwartzman phenomenon. Mean haemorrhagic areas in mm², developed by rabbits prepared with different dilutions of endotoxin. a = controls, b = animals treated with haemocoagulase

Haemocoagulase enhanced the local Schwartzman phenomenon in every case. Each of the rabbits treated with the drug, even those prepared with the smallest dose of endotoxin, developed necrosis.

In vitro haemocoagulase was neither anti nor procomplementary even when used at concentrations which are never reached *in vivo*.

The results suggested that blood clotting plays an important role in the local Schwartzman phenomenon, and the protective effect of heparin seems to be due to its anticoagulant effect. When prescribing haemocoagulase for haemostatic purposes it should be remembered that it may definitely enhance the Schwartzman-type reactions eventually proceeding in the organism.

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IDENTIFICATION OF SHIGELLAE ON THE BASIS OF SELECTIVE SENSITIVITY

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(Received April 26, 1967)

Summary. By use of paper disks impregnated with malachite green, crystal violet, safranin, basic fuchsin, Janus green, cobaltous nitrate and zinc sulphate, characteristic differences have been shown in the sensitivity of various *Enterobacteriaceae* groups. The method is especially useful for the simple and reliable identification of *Shigellae*, which are sensitive to safranin and basic fuchsin.

Culturing on agar plates stained with safranin or basic fuchsin facilitates the identification of *Shigellae* [31]. The paper disk method simplifies the procedure as it requires a single plate for testing the sensitivity to several substances.

Materials and methods

Strains. Partly freshly isolated, partly laboratory strains were examined. The later were the same as in previous investigations [29, 30].

Paper disks, 5 mm in diameter, were prepared from Whatman No.1 filter paper by means of a punching machine. After autoclaving at 121°C for 30 minutes the disks were impregnated with the following solutions (30 disks with 0.15 ml = one disk with 0.005 ml): 1% malachite green (Riedel); 0.5% crystal violet (Chroma); 0.7% safranin T (Miehrome, and KETI, Budapest); 0.25% basic fuchsin (Chroma), 3% Janus green (Reanal, Budapest); 0.7% cobaltous nitrate hexahydrate; 7% zinc sulphate heptahydrate. All compounds were dissolved in boiling water. Janus green solution had been filtered before use. The solutions were storable in a dark place at room temperature for weeks. The impregnated disks were dried at 45°C for 30 minutes and stored in the refrigerator. Their activity did not decrease in 3 months.

The inhibitory substances and their concentrations were chosen in preliminary experiments. At concentrations lower than the optimal the selective inhibitory action was not adequately reproducible; at higher concentrations the selectivity decreased.

Sensitivity testing was carried out similarly to antibiotic susceptibility examinations. One ml amounts of 4 to 5 hour broth cultures were pipetted on agar plates dried previously at 45°C for 30 minutes; after the removal of the excess suspension the disks were placed on the plates. Readings were performed after incubation overnight.

Preliminary experiments indicated that the components of the agar medium were considerably influencing the result. The best results were obtained with the following medium: beef extract, 1.5%; NaCl, 0.3%; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.4%; Richter peptone, 1%; agar, 1.7%; pH 7.4—7.5. Into each Petri dish (90 mm in diameter) 25 ml of medium were poured.

In antibacterial effect the tested substances were considerably inferior to antibiotics and the inhibition zones produced by them were, with the exception of Gram positive bacteria such as *Staphylococcus*, smaller than the zones usually observed around antibiotic disks. In reading the results there was no need to determine the zone diameter. The culture was regarded sensitive to a given substance when growth inhibition occurred around the corresponding disk. Readings were made best at oblique illumination by a hand lens.

Results

Diagnostic value of selective sensitivity. Table I shows results obtained with 1171 strains. Most sensitive organisms among *Enterobacteriaceae* belonged to *Shigella*. These bacteria were inhibited generally by all substances shown in Table I. Safranine and basic fuchsin sensitivity was particularly

Table I
Selective sensitivity in the family *Enterobacteriaceae*

Group (type)	No. of strains	No. of strains sensitive to					
		CV	Sa	BF	JG	Co	Zn
<i>E. coli</i> 055	29	29	1	—	24	—	—
„ 0111	69	69	—	—	24	—	—
„ 0124	51	51	2	—	51	—	—
„ type strains	36	36	1	—	32	10	—
<i>E. alkal. -dispar</i>	8	8	6	4	8	—	—
<i>Klebsiella</i>	59	5	—	—	5	—	—
<i>Citrobacter</i>	30	5	—	—	25	3	—
<i>Enterobacter</i>	36	6	—	—	7	—	—
<i>Serratia</i>	34	24	—	—	7	6	—
<i>S. paratyphi-A</i>	9	1	—	—	1	2	—
<i>S. paratyphi-B</i>	27	6	—	—	8	—	—
<i>S. typhi-murium</i>	170	115	—	—	94	18	—
<i>S. typhi</i>	54	8	—	—	8	54	—
<i>Arizona</i>	21	11	—	—	4	—	—
<i>Sh. dysenteriae</i> 1-10	38	38	35	38	38	33	—
<i>Sh. flexneri</i> 1	65	65	63	65	65	60	65
„ 2	60	60	52	60	60	50	60
„ 3	75	75	64	75	75	55	75
„ 4	26	26	25	25	25	14	26
„ 5	7	7	5	7	7	7	7
„ 6	28	28	26	25	21	28	—
<i>Sh. boydii</i> 1-15	33	33	33	32	33	17	—
<i>Sh. sonnei</i>	45	45	43	44	45	27	—
<i>P. vulgaris</i>	35	35	—	—	—	6	—
<i>P. mirabilis</i>	26	26	—	—	—	7	—
<i>Morganella</i>	30	28	—	—	—	—	—
<i>Retterella</i>	27	27	—	—	—	13	—
<i>Providencia</i>	43	43	—	—	—	12	—

Abbreviations: BF = basic fuchsin, Co = cobaltous nitrate, CV = crystal violet, JG = Janus green, Sa = safranine, Zn = zinc sulphate

characteristic of *Shigella*. *Sh. flexneri* types 1–5 were sensitive also to zinc sulphate. Safranin and basic fuchsin inhibited the growth of *Shigella* strains in 91%. Safranin resistance was shown mainly by *Sh. flexneri* types 2 and 3 (14% of the cultures). It should be noted that the irregular behaviour to safranin of some strains causes no diagnostic difficulty, because all these strains are sensitive to fuchsin and zinc sulphate.

The intensity of inhibition and the character of the zone also help in the differentiation of *Shigellae*. Malachite green inhibited the growth of all bacteria; however, the intensity of inhibition varied with different groups. The average diameter of the zone around the malachite green disk was 15–17 mm for *Sh. dysenteriae*, *Sh. flexneri* and *Sh. boydii*, 13 mm for *Sh. sonnei*, 14 mm for *E. alkalescens-dispar*, 11–13 mm for *E. coli*, 9–10 mm for *Proteus* and *Providencia*, 11 mm for *Serratia* and *S. typhi* and 8–9 mm for other *Enterobacteriaceae*. About half of *Sh. dysenteriae* 2 and *Sh. flexneri* 1–5 strains showed “triple ring” formation around the malachite green disk, and about half of *Sh. flexneri* 1b cultures around the basic fuchsin disk. The phenomenon, probably due to impurities in the substance, was characterized by a narrow ring-shaped growth at the periphery of the inhibition zone. The second ring was surrounded by another, very narrow zone of inhibition.

Table I indicates that the method allows the differentiation of a distinct group of crystal violet sensitive enteric bacteria (*Shigella*, *Escherichia* and *Proteae*). *S. typhi* was characterized by sensitivity to cobaltous nitrate; members of the tribe *Proteae* were distinguished by Janus green resistance.

Reproducibility of the results. A great number of *Shigella* and other enteric bacterial strains was tested for selective sensitivity several times, many of them at four week intervals. The results were reproducible if technical errors could be avoided. The use of 25 ml beef-extract agar poured in 90 mm Petri dishes, the thorough drying of the plates as well as preculturing for 4–5 hours in broth and keeping off bacterial contamination were important requirements.

Antagonists of the inhibiting substances. There are substances (magnesium ion, cysteine, histidine) which antagonize the antibacterial effect of heavy metals. Antagonism can be examined (a) by incorporating the antagonist into the agar; (b) by examining the inhibitory effect of disks impregnated with a mixture of the antibacterial and the antagonistic substance; (c) by placing the disks containing the two substances separately one over the other onto the agar plate.

The above variations were tested for 5 strains of each *Enterobacteriaceae* group. L-cysteine and L-histidine at 2×10^{-6} M antagonized in all experiments the effect of 8×10^{-6} M cobaltous nitrate, but did not influence the inhibitory action of zinc sulphate and dyes. Magnesium sulphate at higher concentrations (10^{-4} M) somewhat decreased the inhibitory effect of certain dyes

(basic fuchsin and Janus green) and of zinc sulphate but was ineffective against cobaltous nitrate.

Substances unsuitable for differentiation. Some of the substances tested (1% acriflavine, 0.5% brilliant green, 3% cobaltous sulphate and 3% cobaltous chloride) inhibited the growth of all members of *Enterobacteriaceae*. The other 27 dyes under examination (e.g. Congored, methylene blue, neutral red) exerted no inhibitory effect even at high concentrations.

Discussion

The selective antibacterial effect of dyes is extensively used for the preparation of culture media. Dye sensitivity testing for identification purposes has, however, been performed only for the differentiation of *Brucella* types. Several decades experience has shown the reliability of HUDDLESON's test [4, 6, 10–16, 19–23, 28, 32, 34, 35]. Our results indicate that testing of selective sensitivity offers a simple, rapid and reliable method for the identification of *Shigella* cultures.

Studies on the mechanism of selective antibacterial inhibition have shown that the majority of substances (salts of heavy metals, basic dyes) act as cationic agents. Positively charged cations are easily adsorbed on the negatively charged microbes. The higher the dissociation (ionization or stabilization constant) of the antibacterial substance, the more effective the drug. The selective antibacterial activity is explained by the fact that the values for these constants vary with different bacteria. Owing to the higher permeability of their cell wall, the inhibitor passes more easily through the cell wall of, and accumulates in, sensitive bacteria. In the cell, the cationic dye is bound by the phosphoric acid group of nucleic acids. Most dyes are salts and in the above characters they resemble, or are actually, salts of heavy metals (for example, malachite green is a double chloride zinc salt of tetramethyl diamino-fuchsonium). The adsorption of dyes takes place according to the mechanism of ion exchange. Between some cations (Mg^{++} and heavy metallic ions) there is a competition for the adsorption to the negatively charged phosphoric acid group of nucleic acids. Cations of heavy metals combine with groups essential in the metabolism of the cell (Hg^{++} binds SH groups, Co^{++} decreases the synthesis of cyanide-sensitive haemenzymes, Zn^{++} inhibits esterase activity). The antibacterial effect decreases in the presence of substances producing complexes with cations. This explains the antagonistic effect of cysteine, the SH group of which combines with Hg^{++} . In addition to cysteine, histidine is also able to decrease or revert the growth inhibitory effect of Co^{++} [1–3, 7–9, 17, 24–27, 33, 36].

Our results are in harmony with the above considerations. That the inhibitory effect is due to cations is indicated by the fact that cobaltous

chloride acted similarly to cobaltous nitrate. There were structurally similar pairs of substances of which only the basic dye was effective (basic fuchsin — acid fuchsin, malachite green — light green). Dyes which have been found suitable for selective sensitivity testing were invariably basic, while the majority of ineffective substances were acid compounds. The antagonistic effect of L-cysteine and L-histidine to cobaltous nitrate also agrees with data in the literature. By forming complexes with cations, these amino acids presumably eliminated the growth-inhibiting effect of metallic ions.

The antagonistic effect of amino acids to the inhibitors indicate that the composition of the medium may considerably influence the result. Cations of metals used in our experiments correspond to trace elements which are able to act at very low concentrations. The fact that the amino acid content of different peptones may vary considerably, is even more important. For example, the histidine content is 0.56% in Richter peptone, 3.1% in Bacto peptone (Difco), and 9.4% in Casamino acids (Difco) [18]. It is obvious that growth inhibition should vary widely with the peptone used for the preparation of the medium.

Acknowledgements. The author is indebted for the *Enterobacteriaceae* strains to DR. V. KERTESZ, Institute of Microbiology, Faculty of Hygiene, Charles University, Prague; DR. R. ALDOVÁ, Institute of Epidemiology and Microbiology, Prague; DR. F. KOLTA Chief, Public Health Laboratory, Tatabánya; to DR. G. HABÁN, Chief, Department of Bacteriology, DR. H. MILCH, Head, Phage Laboratory; DR. B. LÁNYI, Head, Culture Collection; and to DR. S. Z. DEÁK and I. GADÓ, Phage Laboratory, National Institute of Public Health, Budapest.

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CHANGE IN THE PHAGE TYPE OF STAPHYLOCOCCUS AUREUS AS A RESULT OF LYSOGENIZATION

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Summary. Hospital screening tests yielded a number of untypable staphylococcal strains in addition to the common types 29/52/52A/80/52B and 52B. Examination with phages isolated for lysogenization and "prophage-typing" revealed that most untypable strains were identical in typing pattern.

Lysogenization of strains 29/52/52A/80/52B and 52B with phages isolated from untypable strains produced cultures resistant to the basic set of phages.

By use of phages obtained in the experiments the isolated strains were divided into further groups. Induced lysogenization yielded a basis for assuming the mode of formation of untypable strains.

Numerous data have been published on epidemic *Staphylococcus aureus* strains. Of these, type 80/81 was especially common in the last decade, causing severe outbreaks from Australia to Canada [1—3]. Later, other epidemic strains became frequent [4—11]. At the beginning of the sixties' biochemically identical phage resistant strains have been incriminated in various epidemics in England, Canada and the United States. JEVONS and PARKER [16] demonstrated that these strains were derived from phage type 83A: the prophage induced by lysogenesis was built into the cell and blocked the lysis by phage 83A.

Due to the high lysogenicity of, and the multiple *in vivo* and *in vitro* lysogenization inducible in, staphylococci, phage interference arises, influencing phage susceptibility.

Investigations into the lysogenic properties of types 80/81 and 83A clearly demonstrated the association between the blocking prophages and the phage type.

The phage type of strains belonging to the 52, 52A, 80, 81 complex is not quite stable; it varies with the prophage group of these organisms. ROUNTREE [17] and ROUNTREE and ASHESHOV [18] showed that by losing or gaining prophages, strains sensitive to one of the above phages were converted into one of the other types within the group. By analysing the results of several laboratories, ASHESHOV and WINKLER [19] have finally thrown light on these findings. The defective prophage in phage type 80/81 blocks susceptibility to phages 52 and 52A. After losing this prophage or its substitution with another one, the spectrum of the culture becomes wider. A serogroup F pro-

phage, which is present in all members of complex 52, 52A, 80, 81, is also involved. WINKLER *et al.* [20] demonstrated that after losing the serogroup F prophage the spectrum of 52, 52A, 80, 81 strains widened further and the cultures became susceptible to phages of group III. From untypable strains deriving from type 83A, JEVONS *et al.* [21] isolated non-lysogenic variants which were sensitive to phage 83A. When phage type 83A strains were lysogenized with phages released by lysogenic variants, the cultures became untypable.

Experiments performed *in vitro* also indicate that in the fluctuation of the phage type of epidemic strains lysogenization *in vivo* plays a considerable part. Lysogenicity studies on strains isolated in maternity and paediatric wards in Szeged revealed that, by use of suitable phages, strain 52B could be converted into I/52B and *vice versa*. Recent screening examinations showed beside the above types the occurrence of an untypable strain which was lysed only by our phages 162' and 949'. Other examinations seemed to indicate that the three strains differing in phage pattern were related and the difference was due to the presence of blocking prophages.

Materials and methods

Strains. All cultures were isolated in the course of screening tests performed in a maternity unit. Samples were taken from the staff, mothers, babies, and various objects in the wards.

Culturing. The samples were inoculated into 4 per cent potassium rhodanate broth. Next day the enrichment medium culture was streaked on blood agar plates.

Medium for phage experiments. Witte peptone, 1%; beef extract (Central Slaughterhouse, Budapest), 0.75%; agar 1.2%. The pH was adjusted to 7.2 with sodium carbonate.

Phage typing was carried out by the method of BLAIR and WILLIAMS [23] with the following phages. Basic set: 29, 52, 52A, 79, 80, 3A, 3B, 3C, 55, 71, 6, 7, 42E, 47, 53, 54, 75, 77, 83A, 42D, 81, 187; additional set: 42B, 47C, 52B, 69, 73, 78, D, B5, 77AD. The "experimental phages" isolated for lysogenization were also used (Table III).

The "experimental phages" were sterilized by chloroform and used at 1000 RTD. Serological grouping was performed with sera A, B and F. Except for phage 945' (group F), all phages belonged to group B.

Antibiotic sensitivity was tested with penicillin, streptomycin, chloramphenicol, tetracycline, neomycin, polymyxin B, and erythromycin disks (Institute for Serobacteriological Production and Research "Human", Budapest).

Lysogenicity tests and prophage typing were performed as described in reference 22.

Lysogenization. Strains found suitable with prophage typing were heated at 56°C for 2 minutes. The released phages were used for lysogenization either directly or after propagation on suitable cultures. The titre of "experimental phages" ranged between 10^5 to 10^8 PFU/ml. Cultures to be lysogenized were spread on nutrient agar and spotted with the "experimental phages". Secondary colonies within the plaques were streaked on agar plates. The culture was checked for immunity against the lysogenizing phage. The identity in lytic spectrum of the phage released from the lysogenized strains with the lysogenizing phage was also examined. Only those strains which corresponded to the above requirements were considered artificially lysogenized cultures.

Results

Group and type distribution of the 60 *Staph. aureus* strains examined is presented in Table I.

Table I
Distribution of Staph. aureus strains

Group	No. of strains	Phage type	
		RTD	1000 RTD
1	20	52B/29/52A	52/80
2	21	52B	
3	19	NT/162/949/	

Antibiotic sensitivity. The majority of the strains was sensitive to chloramphenicol and neomycin. Some strains were resistant to chloramphenicol. Some chloramphenicol and neomycin sensitive strains were sensitive to penicillin. The antibiotic sensitivity of the strains was unaffected by lysogenization.

Lysogenicity testing indicated that all strains released phages. The lytic spectrum of the temperate phages was generally characteristic of the corresponding phage type. Results are shown in Table II. The lytic spectrum of temperate phages released by strains 29/52/52A/80/52B and 52B was identical but narrower than the lytic spectrum of those obtained from strains 162/949. Some of the former strains produced phages the lytic spectrum of which was as wide as that of strains 162/949.

Table II
Lytic spectrum of temperate phages released from the examined strains

Group	Phage type	Indicator strains										
		6	7	47	54	75	83A	73	52B	D	B _s	162
1	29/52A/ 52/80/52B	++	++	++	—	—	—	—	—	++	++	
2	52B	++	++	++	—	—	—	—	—	++	++	—
3	162/949	++	++	++	++	—	++	++	++	++	+	—

Phages released by heating were spotted without dilution on the indicator strains.

++ = more than 50 plaques.

Lysogenization was performed with phages of strains 162/949 (644, 749, 800) and with phages of strains 52B and 29/52/52A/80/52B which, unlike other phages in this group, exhibited a wide lytic spectrum and lysed propagating strain 52B (741, 783, 822, 845, 874). Phages 945' and 949' described in a previous paper [22] and a phage obtained from strain 162 were also used. The properties of the phages are shown in Table III.

Although the "experimental phages" were isolated from various strains and they differed somewhat in their lytic spectrum, they behaved similarly concerning their ability to lysogenize and to convert the phage type by lysogenization. On the basis of these properties they were divided into three groups (x, y and z).

Table IV summarizes the behaviour to 5 basic and 11 "experimental" phages of the original strains and their variants induced by lysogenization with various phage-group phages.

Lysogenization experiments were performed with 4 representative strains of each of the two phage types (29/52/52A/80/52B and 52B). As the same phage types reacted similarly with the same phage groups, for the sake of simplicity, only one representative of each phage type and only phages exerting lytic activity are shown. With respect to lysogenic response occurring between the examined strains and the "experimental" phages, the latter were divided into groups x, y and z. The original and artificially lysogenized strains were classified into phage types I, X, Y and Z according to their behaviour to basic (they were sensitive only to group I basic phages) and to "experimental phages". This classification allowed a finer grouping of untypable strains. Cultures susceptible to phages x were designated as X, those lysed by phages x and y were named phage type XY, etc.

The lytic pattern of phage type 29/52/52A/80/52B and 52B strains widened if typing was performed with "experimental" phage groups x, y and z; thus they were classified as phage type IXYZ. Untypable strains were lysed by one of the "experimental phage groups" only, according to the phage group of the blocking prophage. Strains belonging to phage types X, XY and XZ were also isolated. By artificial lysogenization in one or more steps all possible patterns could be produced from the original strains, including typing patterns isolated from the examined material. The relationship between the isolated strains and changes developing in artificial lysogenization are summarized in Fig. 1. The relationship between phage types 52B and 29/52A/80/52B described in our previous studies is also included. It is seen that every possible phage type can be induced, except XY which occurred occasionally among 162/949 strains isolated in the course of screening tests. This type may have been induced by a phage other than our "experimental phages".

Incorporation in staphylococci of the lysogenizing phage as a prophage has been proved by the re-isolation of the phage and examination of its lytic spectrum. The lysogenizing phage released after primary lysogenization retained its original lytic spectrum. As compared to the lytic spectrum of temperate phages of the original strains (Table II), the lytic spectrum of phages x, y and z was wider (Table III). As the latter concealed the original spectrum, there was no possibility to determine whether the new phage had been incor-

Table III
Lytic spectrum of "experimental" phages

Phage	Phage type of host	Propagating strain	Serogroup of phage	Designation of group	Indicator strains										
					6	7	47	54	75	83A	73	53B	D	B ₅	162
162'	mixed	7	B	x	++	++	++	++	+	++	+	++	++	—	++
949'	NT	7	B	x	++	++	++	++	—	++	++	++	++	—	++
644'	162/949	52B	B	y	++	++	—	++	—	++	—	++	++	+	—
741'	52B	52B	B	y	++	++	++	++	—	++	—	++	++	++	—
749'	162/949	52B	B	y	++	++	++	—	—	++	+	+	++	+	—
800'	162/949	52B	B	y	++	++	++	++	—	++	++	++	+	++	—
945'	29/52/52A/80/52B	7	F	z	++	++	+	+	+	++	++	++	+	—	+
783'	29/52/52A/80/52B		B	z	++	++	—	—	—	++	—	++	—	—	—
822'	29/52/52A/80/52B		B	z	++	++	—	—	—	++	—	++	—	—	—
845'	29/52/52A/80/52B		B	z	++	++	++	—	++	++	—	++	—	—	—
874'	29/52/52A/80/52B		B	z	++	++	—	—	—	++	—	++	—	—	—

"Experimental" phages were spotted at 1000 RTD on the indicator strains. ++ = more than 50 plaques; + = 20 to 50 plaques; — = no lysis.

Table IV

Phage typing of original and lysogenized strains by basic and "experimental" phages

	Original phage pattern	Lysogenizing phage group	New designation of phage type	Basic phages					"Experimental" phages										
				I					x		y				z				
				29	52	52A	80	52B	162	949	644	741	749	800	945	783	822'	845	874'
Parent strains	29/52/52A		I/X/Y/Z	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	80/52B																		
	52B		I/X/Y/Z					++	++	++	++	++	++	++	++	++	++	++	++
	NT		X						++	++									
	NT		X/Y						++	++	++	++	++	++					
	NT		X/Z						++	++									
	29/52/52A		I/X/Y/Z	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	80/52B																		
	52B		I/X/Y/Z					++	++	++	++	++	++	++	++	++	++	++	++
		x	Y/Z								++	++	++	++	++	++	++	++	++
Strains modified by lysogenization		x, y	Z												++	++	++	++	++
		x, z	Y								++	++	++	++					
		y	X/Z						++	++					++	++	++	++	++
		y, x	Z												++	++	++	++	++
		y, z	X						++	++									
		z	I/X/Y	++	++	++	++	++	++	++	++	++	++	++					
		z, x	Y								++	++	++	++					
		z, y	X						++	++									

porated in addition to the original prophage or a substitution had taken place. As to secondary lysogenization, the effect was the same when the procedure had been carried out first with z then with x or y, or first with x or y then with z. This is explained by the fact that the wider spectrum of phages x or y conceals the narrower spectrum of phages z. In such cases always the wider spectrum prevails irrespective of whether the incorporation occurred primarily or secondarily. If a substitution occurred with phage z after primary

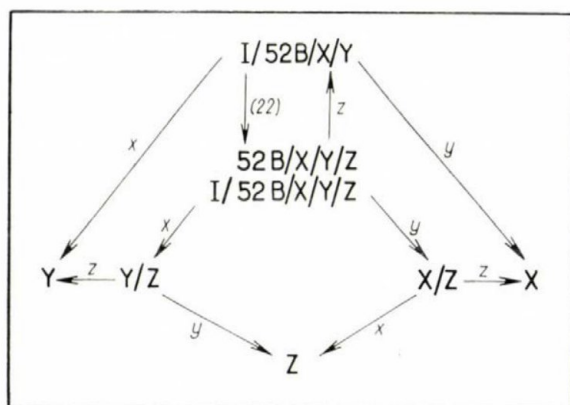


Fig. 1. Phage types induced by artificial lysogenization. x, y, z = groups of lysogenizing phages; I, 52B, X, Y, Z = phage types

lysogenization with x or y, the lytic spectrum of the released phage should be of the narrow type characteristic of z. As the results indicate the presence of wide spectrum phages x or y, it is probable that in addition to phages z incorporated secondarily, primary phages x or y are also present. This finding indicates a double (or, inclusive of the original prophage, a triple) lysogenicity rather than a substitution.

On the basis of the above results it is justified to assume that a similar lysogenization is responsible for the induction of phage resistance in types 29/52/52A/80 and 52B *in vivo*. Such lysogenic conversions are induced most likely by wide lytic spectrum phages such as those present in strains 162/949. Exceptional 52B and untypable strains resembling 162/949 cultures in prophage lytic spectrum release phages which are acting similarly.

Discussion

Staphylococci cultured from the infectious disease, paediatric and maternity wards in Szeged have been phage-typed since 1962. Phage types 52B and 29/52/52A/80/52B were common and can be regarded as nosocomial

strains. They were rarely responsible for severe complications and were isolated mostly from secondary infections in infectious diseases such as measles or influenza. They often caused pemphigoid and conjunctivitis in newborn babies. A more thorough examination of these strains has been carried out since 1964. Biochemical reactions and antibiotic sensitivity testing indicated a probable lysogenic relationship between these strains. Prophage typing gave a further basis for their differentiation. Lysogenization and artificial conversion of the strains were attempted by use of different lytic spectrum phages.

Susceptibility of staphylococcal strains to "experimental phages" and successful lysogenization depend on the strain chosen for the propagation of the "experimental phages". Certain host-range modifications were disadvantageous. For example, phages 783', 822', 845' and 874' of group z were effective only after release, and lost their activity to all strains after propagation on strains 52B, 7 or 162.

In hospital screening tests, mixed phage type strains 52B/29/52A/80/3A/55/71/6/42E/47/53/75/83A/81 were often encountered. This phage type, having a much wider pattern, may have been the precursor strain. Experiments to induce phage types 29/52/52A/80/52B, 52B or untypable strains from this culture with "experimental" and standard phages, were unsuccessful. The mixed phage type was lysogenic itself. Accordingly, if it is involved in the development of the above phage types, it cannot be the parent precursor, which theoretically should not contain a prophage. On the other hand, the mixed phage type may be the precursor of other phage types, in other words it may be converted by lysogenization into phage types I, II or III. Or else, we might even have failed in finding the phage or phages needed for the development (perhaps in several steps) of phage types 29/52/52A/80/52B, 52B and 162/949.

Acknowledgement. The author is indebted to Mr. L. BERÉNYI for collecting the materials and to Miss A. BÖRÖCZ and Miss M. TÓTH for skilled technical assistance.

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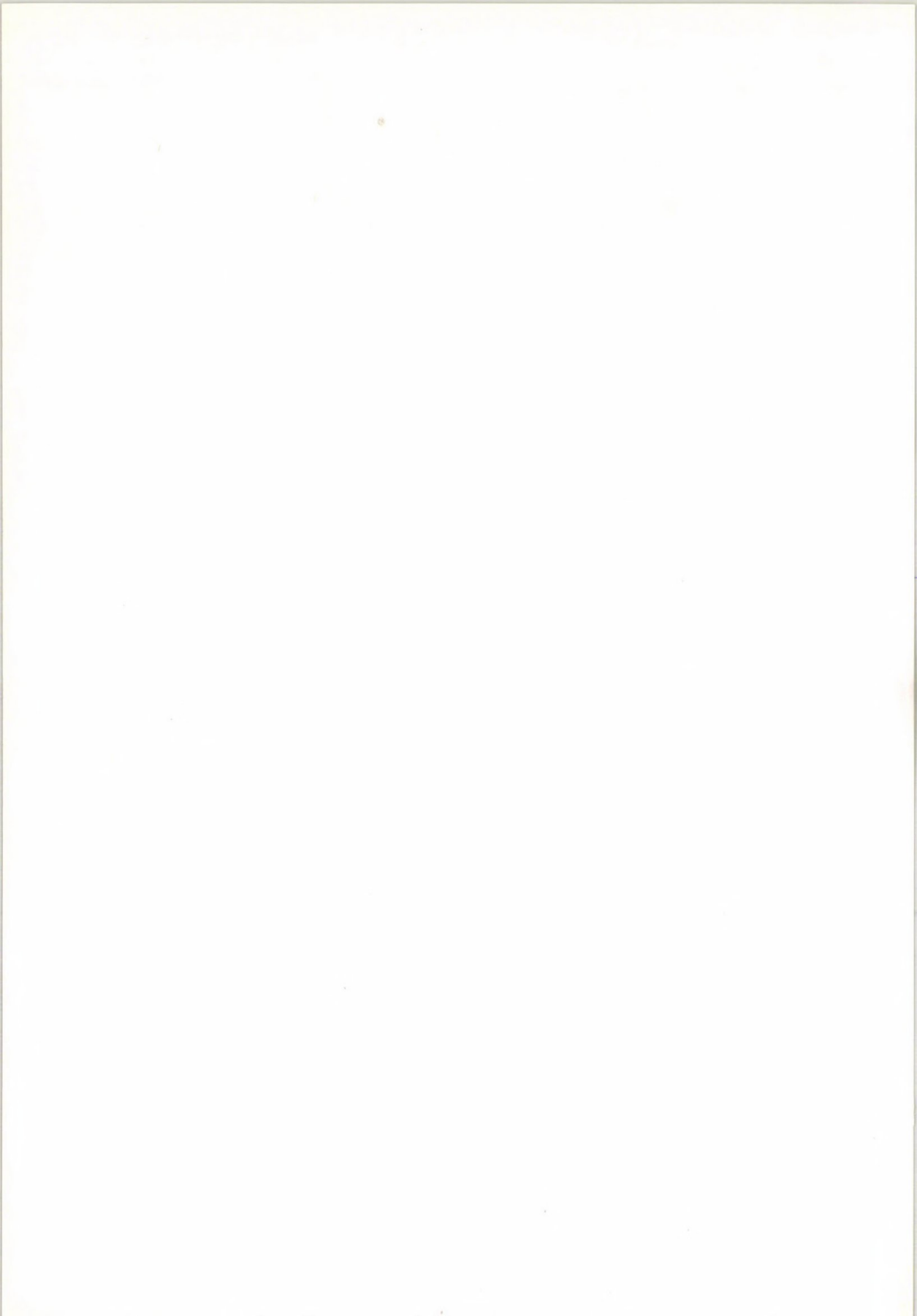
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COMPARATIVE STUDY ON THE INTERFERON PRODUCTION BY THE LEUKOCYTES OF HEALTHY AND LEUKAEMIC SUBJECTS

By

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(Received May 17, 1967)

Summary. (1) Leukocytes obtained from patients with myeloid leukaemia produced more interferon than those from healthy donors. Leukaemic leukocytes infected by Newcastle disease virus produced more interferon than those infected by Sendai virus. Such a difference was not demonstrable when normal leukocytes were used. The average interferon titres were 244 and 38 with the Sendai virus and 985 and 27 with the Newcastle disease virus. Calculated from the average of all experimental results, leukaemic leukocytes produced 15 times more interferon than normal ones.

(2) Interferon production by leukocytes from patients with myeloid leukaemia was less inhibited by actinomycin D than that by normal leukocytes.

(3) Leukocytes from patients with lymphoid leukaemia produced as much or less interferon as those from healthy persons.

Since GRESSER's [1] first studies numerous authors have investigated interferon production by human leukocytes [2, 3, 4, 5, 6].

Recently, the possible viral origin of leukaemias has been raised [7]. Interferon is well-known to influence the course of viral infections in experimental systems [8–11] but its role in the induction and course of tumorous diseases is still obscure.

These considerations have prompted us to compare the interferon-producing capacity of leukocytes from patients with leukaemia with that of normal human leukocytes.

Materials and methods

Preparation of purified leukocyte suspensions. The method evolved by STRANDER and CANTELL [2] was adopted. Fresh citrated blood was centrifuged and the plasma was sucked off. The sediment was resuspended in 1/10 volume of a 5% solution of EDTA and the red blood cells were lysed by adding eight volumes of 0.83% ammonium chloride. The leukocyte suspension thus obtained was incubated at +4°C for 10 minutes, then centrifuged. The supernatant was discarded and the sediment was proceeded as described above. Finally, the leukocytes were resuspended in the desired medium.

Chemical. Actinomycin D (Merck, Sharp and Dohme) 25 mg per ampoule.

Viruses. The Sendai strain of parainfluenza virus type 1 was maintained by intra-allantoic passages in the embryonated egg. Virus-containing allantoic fluids were stored at +4°C.

The H strain of Newcastle disease virus (NDV) was maintained and stored in the same manner. The Indiana strain of the vesicular stomatitis virus (VSV) was propagated in HeLa cell cultures and the virus-containing media were stored at -70°C .

Cell cultures. The continuous U line of human amnion cells was propagated in a medium containing 90 per cent PARKER's medium 199 with 0.125% sodium bicarbonate and 10% calf serum.

Interferon production. Purified leukocytes were suspended, 5×10^6 cells per ml, in a medium of pH 7.4, containing 90 per cent Parker's medium 199 with 0.25% bicarbonate and 10% calf serum. The leukocyte suspension was infected with a multiplicity rate of 1 : 100 with Sendai virus or NDV and then 5 ml volumes were distributed in siliconized centrifuge tubes. The tubes were then stoppered tightly and incubated in a roller apparatus at 18 r.p.m. for 24 to 48 hours.

Interferon assay. After incubation the leukocyte suspension was centrifuged and the supernatant was dialysed against 100 volumes of a glycine-HCl buffer of pH 2 for 72 hours. Subsequently the pH was adjusted to 7.4 by dialysing against phosphate-buffered saline.

Of the preparation to be titrated serial fourfold dilutions were made in a Melnick-Earle solution containing 2% calf serum, 0.5% lactalbumin hydrolysate and 0.125% sodium bicarbonate. Two monolayer tube cultures of U cells were incubated overnight with each of the dilutions, 1 ml per tube. Then the cultures were washed and infected with about 50 CPD₅₀ of VSV in 1 ml of the above medium. Results were read when in the interferon-free control tubes the virus had affected 70 to 100% of the cells. The highest dilution protecting 50% of the cells was regarded as interferon titre.

Results

Tables I and II show the interferon production by leukocytes obtained from two patients with acute and six with chronic leukaemia. Interferon production was induced by Sendai virus and in three cases also by NDV.

Table I

Interferon production by leukocytes from patients with acute myeloid leukaemia and by those from healthy donors

Leukaemic* donors	Healthy donors	Interferon titre after infection with	
		Sendai virus	NDV
T. G.		2000	8000
	L. P.	64	64
G. M.		128	N. T.**
	L. M.	64	N. T.

* = Peripheral smears. T. G.: paramyeloblasts, 59%; promyelocytes, 1%; myelocytes, 3%; young forms, 7%; stab forms, 7%; polymorphonuclears, 19%; lymphocytes, 4%. G. M.: paramyeloblasts, 60%; stab forms, 2%; polymorphonuclears, 16%; lymphocytes, 22%.

** = Not tested

In every case but one the leukaemic leukocytes produced more interferon than those obtained from healthy donors. The excess interferon was the greatest in the case of T. G., a patient suffering from acute myeloblastic leukaemia.

Table II

Interferon production by leukocytes from patients with chronic myeloid leukaemia and by those from healthy donors

Leukaemic donors	Healthy donors	Interferon titre after infection with	
		Sendai virus	NDV
R. S.		64	N. T.*
	V. J.	32	N. T.
Sz. J.		128	N. T.
	F. J.	64	N. T.
M. L.		64	N. T.
	Sz. J.	16	N. T.
F. E.		8	N. T.
	Sz. I.	8	N. T.
T. S.		N. T.	128
P. S.		N. T.	128
	F. I.	N. T.	32

* N. T. = not tested

From four patients leukocytes were repeatedly taken for interferon experiments (Table III). Although the numerical data show a considerable fluctuation, the leukocytes obtained from B. G., T. I. and J. J. always produced

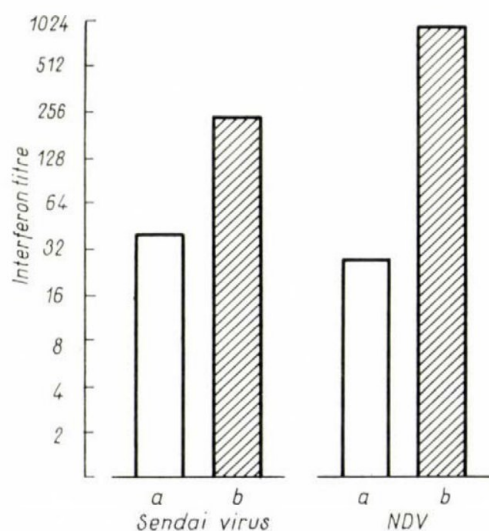


Fig. 1. Comparison of the interferon-producing capacity of leukocytes from patients with myeloid leukaemia (b) and those from healthy donors (a)

Table III

Interferon production by leukocytes obtained from the same donors at intervals of several weeks

Leukaemic donor	Healthy donor	Time interval, weeks	Interferon titre after infection with	
			Sendai virus	NDV
T. L.	V. J.	—	24	N. T.*
			32	N. T.
T. L.	B. I.	5	16	128
			32	16
T. L.	Cs. Cs.	6	128	128
			64	32
B. G.	H. A.	—	512	N. T.
			128	N. T.
B. G.	Cs. Cs.	12	512	128
			64	32
B. G.	N. L.	2	32	128
			<4	16
T. I.	L. J.	—	16	N. T.
			<4	N. T.
T. I.	F. J.	1	128	N. T.
			64	N. T.
T. I.	G. K.	4	128	512
			32	32
J. J.	B. I.	—	256	512
			32	16
J. J.	P. Gy.	1	16	64
			<4	<4

* N. T. = not tested

more interferon than the control leukocytes. The same was observed when interferon production was induced in T. L.'s leukocytes by NDV, while the Sendai-virus-infected leukocytes of the same patient produced approximately as much interferon as the controls.

Fig. 1 shows the average interferon titre for all the myeloid leukaemic leukocytes and that for all the normal leukocytes tested.

NDV-infected leukaemic leukocytes produced, on the average, four times more interferon than those infected with the Sendai virus, whereas interferon production by normal leukocytes appeared independent of the virus that had induced the production.

To clarify the mechanism of the increased interferon production by leukocytes from patients with myeloid leukaemia, we added actinomycin D

Table IV

Effect of actinomycin D on the interferon-producing capacity of leukocytes obtained from healthy donors and from patients with myeloid leukaemia

Leukaemic donors	Healthy donors	Actinomycin D $\mu\text{g/ml}$	Interferon titre
B. G.*	Cs. Cs.	2	<4
		1	128
		0	512
		2	<4
		1	<4
		0	64
M. L.*	Sz. J.	2	32
		1	32
		0	64
		2	<4
		1	<4
		0	16
T. G.**	V. V.	2	8
		1	32
		0	2000
		2	<4
		1	<4
		0	64

* = Chronic myeloid leukaemia

** = Acute myeloid leukaemia

to leukocyte suspensions simultaneously with their infection with the Sendai virus. Table IV shows that 1 or 2 mg of actinomycin D in 1 ml medium completely prevented interferon production by normal leukocytes, whereas inhibition was partial in the case of leukocytes obtained from patients with myeloid leukaemia.

In Table V, interferon production by leukocytes obtained from four patients with lymphoid leukaemia is presented. In these four cases interferon production was not significantly different from the control values; deviations occurred in both directions.

Finally, without viral infection neither the myeloid nor the lymphoid leukaemic leukocytes produced interferon. Interferon-like substance could not be detected in leukaemic sera.

Table V

Comparison of the interferon-producing capacity of leukocytes from patients with chronic lymphoid leukaemia and those from healthy donors

Leukaemic donors	Healthy donors	Interferon titre after infection with	
		Sendai	NDV
K. K.		256	N. T.*
	H. A.	128	N. T.
L. L.		4	16
	Sz. I.	8	8
O. L.		32	8
	Cs. Cs.	64	32
O. L.		<4	16
	L. P.	64	64

* = Not tested

Discussion

The leukocytes of 12 patients suffering from acute or chronic myeloid leukaemia produced substantially more interferon than normal leukocytes. The difference was more pronounced when interferon production was induced by NDV than when it was induced by the Sendai virus. The increased interferon production was consistently demonstrable with leukocytes obtained in intervals of several weeks. Calculated from all the data obtained in the present work, the average interferon level reached 519 and 34 in myeloid leukaemic and normal leukocyte suspensions, respectively. This means that the leukaemic leukocytes produced 15-fold more interferon than the normal ones. Such a difference was not demonstrable for the leukocytes of patients suffering from lymphoid leukaemia.

We could not demonstrate any correlation between the duration of illness and the applied treatment on the one hand and the interferon titre on the other.

We have not found any literary data on the interferon production by leukocytes of patients with myeloid leukaemia. As regards lymphoid leukaemia, LEE *et al.* [12] demonstrated a decreased interferon production by the leukocytes. The present data seemed to agree with these observations.

The increased interferon production by myeloid leukaemic leukocytes might be attributed to an increased inducibility of nuclear RNA synthesis. This idea was further supported by our experiments with actinomycin D. In the case of lymphoid leukaemia an inverse situation might take place as supported

by the data published by TRYFIATES and LASZLO [13], suggesting that nuclear RNA synthesis is different in lymphoid and chronic myeloid leukaemias.

The present results are inconsistent with the general view that tumorous and, in general, undifferentiated cells are weak interferon producers. In this respect the increased interferon production by the leukocytes obtained from two patients with acute leukaemia (G. M. and T. G.) may be mentioned. In the blood of both of these the majority of the leukocytes was represented by paramyeloblasts (60 and 59 per cent, respectively). In the blood of T. G. the percentage of lymphocytes was extremely low (4%). Thus, the increased interferon production may be attributed first of all to immature cells, the paramyeloblasts.

The present data are in contrast with the results of SOLOVIEV *et al.* [14], who found that leukaemic leukocytes produced little, if any, interferon. These authors have assumed some parallelism between interferon production and the general immune status. The divergent results might be attributed to different clinical materials.

Acknowledgements. The authors are indebted to Prof. K. CANTELL (Helsinki, Finland) for the U cell line, to Dr. LILI ASZÓDI (County Hospital, Debrecen) for making available fresh blood samples and to Mr. J. MÁRTON for excellent technical assistance.

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J. HORVÁTH

FUNDAMENTAL GENETICS OF STREPTOMYCETES

*In English · Approx. 150 pages · 28 photos · 22 tables ·
17 × 24 cm · Cloth*

This work summing up some 17 years of the author's research on the genetics of Streptomyces covers practically the whole field of the relevant problems. Topics like the variability depending on the composition and pH of the medium, lasting modification ("Dauermodifikation"), transformation, transduction are dealt with and a detailed description of heterokaryosis and of nuclear position is given in the book. Data are presented about the stabilization and constant prevalence of heterokaryosis. The author provides evidence that Streptomyces have amitotically dividing nuclei of polyploidic nature and that this phenomenon accounts for their great variability.



AKADÉMIAI KIADÓ

PUBLISHING HOUSE OF THE HUNGARIAN
ACADEMY OF SCIENCES
BUDAPEST 502. P.O.B. 24

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója

Műszaki szerkesztő: Farkas Sándor

A kézirat nyomdába érkezett: 1967. X. 14. — Terjedelem: 7,50 (A/5) ív, 31 ábra

67.64562 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

**FORMAMIDE BREAKDOWN REACTION FOR THE DIFFERENTIATION
OF ENTEROBACTERIACEAE**

B. SERÉNY

**ИСПОЛЬЗОВАНИЕ РЕАКЦИИ РАСЩЕПЛЕНИЯ ФОРМАМИДА
ДЛЯ ДИФФЕРЕНЦИРОВКИ ЭНТЕРОБАКТЕРИЙ**

Б. ШЕРЕНЬ

С применением технически простой реакции подщелачивания среди бактерий, относящихся в семью *Enterobacteriaceae*, можно распознать такие штаммы, которые быстро и энергично (все исследованные культуры *Morganella* и часть культур *Klebsiella* и *Rettgerella*) или медленно (*Serratia*) разлагают формамид. Их легко отличить от формамид-негативных кишечных бактерий (прочие генусы семьи *Enterobacteriaceae*). Ни одна из исследуемых кишечных бактерий не разлагала другие химические соединения, содержащие аминогруппу (диметилформамид, пара-аминосалициловая кислота, пара-аминобензойная кислота, β -нафтиламин, о-фенилендиамин, анилин, диэтиламин, уретан, гексаметиленetetрамин).

**ANTIBIOTIC PRODUCTION OF HYPHAL FRACTIONS
OF STREPTOMYCES GRISEUS**

F. SZESZÁK, G. SZABÓ

**ПРОДУКЦИЯ АНТИБИОТИКА ФРАКЦИЯМИ ГИФНА STREPTOMYCES
GRISEUS**

Ф. СЕСАК, Г. САБО

В опыте с коротким инкубационным периодом авторы исследовали отмутый мицелий штамма *Streptomyces griseus* 52—1 с целью измерения степени способности продукции стрептомицина. Было найдено, что активность по продукции стрептомицина можно хорошо измерять при 2—3 часовом встряхивании в дважды разведенной соевом кукурузной питательной среде. Глюкоза в опытных условиях подавляла продукцию стрептомицина.

Определили, в какой степени может мешать измерению стрептомицин-продуцирующей активности стрептомицин, выделенный ранее и адсорбированный на отмутых мицелиях.

Авторами было установлено, что стрептомицин-продуцирующая активность мицелиев после промывки фосфатным буфером равна 30—50% активности мицелиев, размножающихся и далее в первоначальной среде.

Исследовали эффект дезинтеграции, происходящей в смесителе Waring-a, на стрептомицин-продуцирующую активность; на действие дезинтеграции выработка стрептомицина ускоряется.

IMMUNOFLUORESCENT STUDIES ON YEASTS

P. GECK, E. K. NOVÁK

ИММУНФЛЮОРЕСЦЕНТНОЕ ИССЛЕДОВАНИЕ ДРОЖЖЕЙ

П. ГЕКК, Е. К. НОВАК

На основе прямого иммунфлуоресцентного окрашивания конъюгатом *anti-albicans* 107 штаммов дрожжей из 102 видов и проведенного непрямого окрашивания 13 штаммов, принадлежащих к 7 видам, было установлено, что при соответствующем разведении конъюгата или иммунной сыворотки специфичность метода может быть повышена в такой степени, что становится возможным с уверенностью отличать *Procandida albicans* и *Pr. stellatoidea* от других видов дрожжей. На основе сравнения результатов иммунфлуоресценции и диагностических критериев, принятых в настоящее время, авторы считают, что метод иммунной флуоресценции может быть применен для решения вопросов таксономии.

CHROMATOGRAPHIC CHARACTERIZATION OF ECHOVIRUS PROTOTYPE STRAINS

E. SZÖLLÖSY, GY. LENCYEL, É. ÁGOSTON

ХРОМАТОГРАФИЧЕСКАЯ ХАРАКТЕРИСТИКА ПРОТОТИПНЫХ ШТАММОВ ВИРУСОВ ECHO

Е. СЕЛЛЁШИ, ДЬ. ЛЕНДЬЕЛ, Е. АГОШТОН

Авторы изучали элюционные свойства прототипных штаммов вирусов ECHO методом хроматографии на ДЕАЕ-целлюлозном столбе. Элюция происходила при повышающейся молярности NaCl. Штаммы характеризовались теми фракциями молярности NaCl, при которых элюция вируса была наиболее выраженной. На этой основе прототипные штаммы были подразделены на три группы. В первую группу входили штаммы, элюирующие при низкой ($< 0,01$), во вторую — при средней ($0,01-0,09$), в третью — при высокой ($> 0,09$) молярности NaCl. Соответственно этому первая группа включала в себя 1, 8, 12, вторая группа — 1 (второй максимум элюции), 4, 7, 13, 14, 20, 22, 24, 29, третья группа — 2, 3, 5, 6, 9, 11, 15, 16, 17, 18, 19, 21, 23, 25, 26, 27, 30 и 32 прототипные штаммы. Авторы обсуждают значение хроматографических исследований в определении внутритиповых характеристик.

REPLICATION OF HERPES SIMPLEX VIRUS IN ARGININE-FREE MEDIA

I. EFFECT OF ARGININE-DEFICIENCY IN DIFFERENT TISSUE CULTURE CELLS

E. JENEY, É. GÖNCZÖL, L. VÁCI

РАЗМНОЖЕНИЕ ВИРУСА HERPES SIMPLEX В СЛУЧАЕ ИСПОЛЬЗОВАНИЯ ПИТАТЕЛЬНОЙ СРЕДЫ, СВОБОДНОЙ ОТ АРГИНИНА

I. Влияние недостатка аргинина в различных тканевых культурах

Е. ЙЕНЕИ, Е. ГЕНЦЁЛ, Л. ВАЦИ

Размножение вируса *Herpes simplex* в условиях отсутствия аргинина в клеточных культурах HeLa, Her2 и KB подавлялось, в то время как в человеческих и куриных эмбриональных фибробластах, а также в клетках первичной культуры почки обезьяны вирусное размножение при тех же условиях не нарушалось.

Использование питательной среды, свободной от аргинина, не влияло на адсорбцию вируса *Herpes simplex* ни к клеткам HeLa, ни к человеческим эмбриональным фибро-

бластам. В случае этих же культур при отсутствии аргинина не подавлялся и выход вируса из клетки. Повидимому, при отсутствии аргинина тормозится каждый период, следующий за вирусной адсорбцией (пенетрация, репродукция). Удаление аргинина в случае клеток HeLa в одинаковой степени подавляет цитопатические изменения, характерные для вируса, и появление вирусного антигена внутри клетки.

REPLICATION OF HERPES SIMPLEX VIRUS IN ARGININE-FREE MEDIA II. DNA SYNTHESIS IN INFECTED AND NON-INFECTED HUMAN EMBRYONIC FIBROBLAST AND HeLA TISSUE CULTURE CELLS

É. GÖNCZÖL, E. JENEY, L. VÁCZI

РАЗМНОЖЕНИЕ ВИРУСА HERPES SIMPLEX В СЛУЧАЕ ИСПОЛЬЗОВАНИЯ ПИТАТЕЛЬНОЙ СРЕДЫ, СВОБОДНОЙ ОТ АРГИНИНА

II. Синтез ДНК в зараженных и незараженных клетках человеческих эмбриональных фибробластов и HeLa

Е. ГЕНЦЕЛ, Е. ЙЕНЕИ, Л. ВАЦИ

В условиях отсутствия аргинина обмен веществ в клетках HeLa понижается, особенно страдает синтез ДНК. При исследовании с помощью инкорпорированного ^{32}P выяснилось, что недостаток аргинина не вызывает изменений в обмене веществ фибробластов эмбриона человека. При наличии питательной среды, содержащей аргинин, во фракции ДНК клеток HeLa, зараженных вирусом *Herpes simplex*, можно наблюдать усиленную инкорпорацию ^{32}P по сравнению с контролем, тогда как в условиях отсутствия аргинина в активности ДНК зараженных и незараженных клеток HeLa никакой разницы не наблюдалось. Содержание общей клеточной ДНК при исследовании методом по *Burton* не изменялось ни на заражение вирусом *Herpes simplex*, ни на действие питательной среды, не содержащей аргинина.

RELATION OF THE ANTIBACTERIAL ACTIVITY AND CHEMICAL STRUCTURE OF CHALCON DERIVATIVES

M. GÁBOR, J. SALLAI, T. SZÉLL, GY. SIPOS

ВЗАИМОСВЯЗЬ МЕЖДУ БАКТЕРИОСТАТИЧЕСКИМ ДЕЙСТВИЕМ И ХИМИЧЕСКОЙ СТРУКТУРОЙ ПРОИЗВОДНЫХ CHALCON-A

М. ГАБОР, Й. ШАЛЛАИ, Т. СЕЛЛ, ДЬ. ШИПОШ

С помощью метода агарового слоя изучали антибактериальное действие производных Nitrohydroxychalcon-a на штаммы *Staphylococcus albus* и *Staphylococcus aureus*.

Оптимальный бактериостатический эффект наблюдался при размещении гидроксильной группы кетонной части в положении 4'. Производные chalcon-a менее эффективны, если гидроксил находится в положении 3' или 2'. Нитрогруппа при гидроксильной имеет второстепенное значение, хотя несколько повышает эффект. На активность положительно влияет замена галогена в положениях 2 и 4. С этой точки зрения больший эффект наблюдается от флора, чем от атомов брома или хлора. Нафтаальдегид, р-изопропил-бензальдегид и chalcon-ы, полученные из ниперонала, были эффективны во многих случаях, а производные с 3-нитро-салицил-альдегидом во всех случаях.

POST-THERAPEUTIC ANTIBIOTIC CONTENT OF FAECES

S. BOGNÁR, L. BINDER

СОДЕРЖАНИЕ АНТИБИОТИКОВ В ФЕКАЛИЯХ ПОСЛЕ ИХ ТЕРАПЕВТИЧЕСКОГО ПРИМЕНЕНИЯ

Ш. БОГНАР, Л. БИНДЕР

Авторы определяли титр антибиотиков в фекалиях после завершения лечения хлорамфениколом, окситетрациклоном и неомицином, введенных в организм перорально и парентерально. Спустя 24 часа после парентерального введения в стуле ни один из трех антибиотиков не был определен. В случае перорального введения определить в фекалиях хлорамфеникол через 24 часа после его дачи — подобно парентеральному введению — не удалось. Окситетрациклин, данный перорально, после завершения лечения исчез из стула каждого обследованного больного на 5 день, а неомицин на 7 день. Считая от завершения лечения, окситетрациклин на 4 день, а неомицин на 5 день ещё мог быть определен в терапевтических значениях в стуле половины обследованных больных.

DEFEKTIV LYSOGENE BACILLUS ANTHRACIS-STÄMME

G. Ivánovics

ДЕФЕКТИВНЫЕ ЛИЗОГЕННЫЕ ШТАММЫ BACILLUS ANTHRACIS

ДЬ. ИВАНОВИЧ

Автору удалось выделить дефективные лизогенные бактерии из культуры оболочковой *Bacillus anthracis*, зараженной вирулентным мутантом W-фага и содержащейся в атмосфере CO₂. Из этих безоболочковых мутантов, несущих профаг, фаг не освобождался, и только на основе иммунитета против W-фага можно было судить об их лизогенных свойствах. Гомологичный фаг адсорбировался на эти дефективные лизогенные штаммы, в то время как на фагрезистентные — нет. Удалось выделить темперируемые и вирулентные мутанты W-фага, которые соответственно с дефективными штаммами *B. anthracis* образуют лизогенный комплекс или растворяют последние.

FATTY ACID COMPOSITION OF STAPHYLOCOCCUS AUREUS STRAINS DIFFERING IN ANTIBIOTIC SENSITIVITY

I. RÉDAI, A. RÉTHY, L. VÁCI

СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ ЖИРНЫХ КИСЛОТ ШТАММОВ STAPHYLOCOCCUS AUREUS С РАЗЛИЧНОЙ ЧУВСТВИТЕЛЬНОСТЬЮ К АНТИБИОТИКАМ

И. РЕДАИ, А. РЕТИ, Л. ВАЦИ

Авторы изучали общее содержание и спектр жирных кислот штаммов *Staphylococcus aureus*, различно чувствительных к антибиотикам.

Было установлено, что общее содержание жирных кислот чувствительных и резистентных к антибиотикам штаммов *Staphylococcus aureus* существенно не отличается. Штаммы *Staphylococcus aureus* с различной чувствительностью к антибиотикам синтезируют одинаковые жирные кислоты; основные компоненты спектра полученных жирных кислот: anteiso C₁₅, арахиновая кислота, жирная кислота, содержащая C₁₇-циклопропановое кольцо или разветвленную углеродную цепочку, и стеариновая кислота.

Процентное распределение жирных кислот, полученных после 18-часового культивирования в строго одинаковых условиях штаммов с различной чувствительностью к антибиотикам, оказалось различным. 16—20% жирных кислот штаммов, чувствительных

к антибиотикам, состояли из циклопропанового кольца с 17 и 19 углеродными атомами или представляли собой жирные кислоты с разветвленной углеродной цепочкой; в то же время пропорция этих кислот в спектре жирных кислот резистентных штаммов была равна 34—44%.

STUDIES ON THE VIRULENCE OF SHIGELLA FLEXNERI

K. RAUSS, I. KÉTYI, T. ANGYAL

ИЗУЧЕНИЕ ВИРУЛЕНЦИИ ШТАММОВ SHIGELLA FLEXNERI

К. РАУШШ, И. КЕТЬИ, Т. АНДЬЯЛ

Авторы на основе расхождений в преломлении света колониями выделили вирулентные и авирулентные клоны из штаммов *Shigella flexneri* 2a и 3 типа. Исследование этих, а также нескольких мутантов, выделенных исследовательской группой Walter Reed-a, далее — стрептомицин-зависимого аттенуированного штамма Mel-a было проведено на основе следующих точек зрения: характер роста, способность вызывать кератоконъюнктивит у морских свинок, патогенность для мышей, инфекционность для куриных эмбрионов при внутривенном и аллантоисном введении. Часть клонов оказалась вирулентной, в то время как другая часть проявляла авирулентные свойства. Авирулентные клоны удалось подразделить в 3 группы: совершенно авирулентные мутанты; клоны, утратившие способность к пенетрации; группа, представленная гибридным штаммом Formal-a и сотрудников, который сохранил способность к пенетрации, но не способен размножаться *in vivo*.

IMMUNOGENICITY

OF ORALLY ADMINISTERED AVIRULENT SHIGELLA CLONES

K. RAUSS, I. KÉTYI, T. ANGYAL

ИММУНОГЕННОСТЬ АВИРУЛЕНТНЫХ КЛОНОВ SHIGELLA В СЛУЧАЕ ПЕРОРАЛЬНОГО ВВЕДЕНИЯ

К. РАУШШ, И. КЕТЬИ, Т. АНДЬЯЛ

По ходу изучения в опытах на животных аттенуированных штаммов *Shigella*, относящихся к различным авирулентным категориям, было установлено, что те авирулентные мутанты, которые утратили способность к пенетрации, не иммунизируют эффективнее, чем вакцина, содержащая убитые бактерии. Иммуногенность гибридного штамма, полученного исследовательской группой Walter Reed-a и располагающего способностью к пенетрации, превышает таковую других авирулентных штаммов и иммуногенность вакцин, содержащих убитые бактерии, но практически не превышает иммуногенность антигена Boivin. Совершенно безопасный антиген Boivin в настоящее время может рассматриваться как наиболее приемлемый (его применяют и *per os*).

Авторы обсуждают вопрос о возможностях и ограничениях применения аттенуированных штаммов *Shigella*.

MAINTENANCE OF ARTIFICIAL DYSENTERY IMMUNITY BY ORAL VACCINATION IN HUMANS

K. RAUSS, S. PUSZTAI, I. JOÓ, I. KÉTYI, J. MÁTÉ

ПОДДЕРЖАНИЕ В ЧЕЛОВЕКЕ ИСКУССТВЕННОГО ИММУНИТЕТА ПРОТИВ ДИЗЕНТЕРИИ ПУТЕМ ПЕРОРАЛЬНОЙ ВАКЦИНАЦИИ

К. РАУШШ, Ш. ПУСТАИ, И. ЙОО, И. КЕТЬИ, Й. МАТЕ

Из диализированного Boivin-экстракта штаммов *Shigella flexneri* 2a и 3, а также *Shigella sonnei*, которые сохранили свою иммуногенность после обработки алкоголем и лиофилизации и не оказались токсичными, была приготовлена пероральная вакцина.

Лица, получившие основной иммунитет путем повторной парентеральной прививки адсорбированной вакциной, а также ревакционированные лица спустя месяц после парентеральной иммунизации в течение 8—12 недель были еженедельно вовлечены в пероральную ревакцинацию.

На основе попыток пассивной защиты мышей, проведенных с помощью муцинтехники, можно было установить, что пероральная ревакцинация поддерживает в мышках защитный уровень антител, возникших на действие парентеральной иммунизации, тогда как при отсутствии ревакцинации уже через 3 месяца наблюдается довольно значительное понижение титра.

Эффект от пероральной ревакцинации был подтвержден и в опыте, проведенном на модели «мышь-шингеллез».

ADJUVANT EFFECT OF STREPTOMYCIN ON ORAL IMMUNIZATION WITH SHIGELLA ANTIGENS IN THE MOUSE

I. KÉTYI, K. RAUSS

АДЪЮВАНТНОЕ ДЕЙСТВИЕ СТРЕПТОМИЦИНА ПРИ ПЕРОРАЛЬНОЙ ИММУНИЗАЦИИ МЫШЕЙ АНТИГЕНАМИ SHIGELLA

И. КЕТЬИ, К. РАУШШ

В мышках, получавших стрептомицин, иммунный эффект от пероральной вакцины, содержащей убитые формалином бактерии или состоящей из экстракта Boivin, возрос на 2—3 степени. Одновременное применение стрептомицина с антигеном также оказалось эффективным, и интенсивность адъювантного действия находилась в тесной зависимости от примененной концентрации стрептомицина.

THE FERTILITY OF ESCHERICHIA COLI 0101 : K(A)30: — STRAIN 18A10 IN MATING WITH ESCHERICHIA COLI K12

I. KÉTYI, L. SZENDREI

ИССЛЕДОВАНИЕ ПЛОДОВИТОСТИ ESCHERICHIA COLI 0101 : K(A)30:, ШТАММ 18A10, С ESCHERICHIA COLI K12

И. КЕТЬИ, Л. СЕНДРЕИ

Из патогенного для мышей-сосунков штамма *E. coli*, обозначенного «18A10», с антигенным строением 0101 : K30: авторам удалось получить мутанты, располагающие подходящими маркерами. На основе анализа конъюгации, проведенного против системы *E. coli* K12, штамм оказался F⁻ характера. Из 6 изучаемых маркеров (пролин, треонин, лактоза, метионин, гистидин и стрептомицин) конъюгацией могли быть перенесены маркеры пролин и треонин, тогда как метионин, гистидин и стрептомицин — нет. Перенос области лактозы удавался только с низкой частотой и частично в нестабильной форме. Так, штамм *E. coli* 0101 : K30:, обозначенный «18A10», оказался плодородным от *E. coli* K12, но между двумя штаммами имеется генетическая ингомлогичность большой степени.

SEGREGATION OF R FACTOR IN SHIGELLA AFTER ACRIDINE ORANGE TREATMENT

I. KÉTYI, A. VERTÉNYI

СЕГРЕГАЦИЯ R-ФАКТОРА В СЛУЧАЕ SHIGELLA ПОСЛЕ ОБРАБОТКИ АКРИДИНОРАНЖЕМ

И. КЕТЬИ, А. ВЕРТЕНЬИ

У штамма *Shigella sonnei*, резистентного к сульфонамиду, стрептомицину, хлорамфениколу и тетрациклину, обработка акридиноранжем обусловила сегрегацию. Сегрегационные клоны были чувствительны к стрептомицину-хлорамфеникол-тетрациклину

или только к тетрациклину. По мнению авторов, резистенция штамма к сульфонамиду или хромосомного происхождения, или является результатом рекомбинации сульфонамид-детерминанты R-фактора и гомологичной хромосомы. Наблюдаемая сегрегация R-фактора подтверждает существование двух linkage-групп в R-факторе. Результаты, полученные при повторной обработке акридиноранжем с применением ультрафиолетового облучения, предоставляют дополнительные данные об интеграции R-фактора.

MORGANELLA MORGANII ANTIGENICALLY RELATED TO ESCHERICHIA COLI 0111 : K58(B4)

B. RALOVICH, S. VÖRÖS

АНТИГЕННОЕ РОДСТВО ШТАММА MORGANELLA MORGANII С ESCHERICHIA COLI 0111 : K58(B4)

Б. РАЛОВИЧ, Ш. ВЕРЁШ

Было показано близкое антигенное родство между штаммом *Morganella morganii* 8662/64 и серогруппой *E. coli* 0111. Установлено, что антиген «b» штамма *E. coli* 0111a,b содержит два фактора («b1» и «b2») и что O антиген штамма *Morganella morganii* подобен «a» и «b2» антигену *E. coli* 0111.

Антиген K58(B4) также оказался комплексным. В штаммах типа 0111a,b и 0111a,c наряду с общим антигеном «Ba» можно обнаружить менее развитые антигены «Bb» и «Bc». Кроме того, был найден и антиген «Bd», носящий характер агглютиногена.

Штамм *Morganella morganii* 8662/64 содержал «Ba» и «Bd» компоненты антигена K58(B4). Температурная чувствительность K антигена *Morganella morganii* оказалась схожей с таковой B антигена *E. coli*. Два антигена, однако, по функции существенно отличались один от другого, так как K антиген *Morganella morganii* агглютинабельность не подавлял, а усиливал. Штамм 8662/64 представляет собой новый серотип *Morganella morganii*, который в антигенной схеме получил обозначение «34».

FIVE NEW SEROTYPES OF MORGANELLA MORGANII

K. RAUSS, S. VÖRÖS

ПЯТЬ НОВЫХ СЕРОТИПОВ MORGANELLA MORGANII

К. РАУШШ, Ш. ВЕРЁШ

Авторы сообщают о биохимических исследованиях и изучении антигенной структуры пяти новых серотипов *Morganella morganii*. Предлагают, чтобы штаммы как представители 30—34 серогрупп образовали продолжение антигенной схемы, ранее разработанной авторами.

Предлагают штаммы 958/018 : H (18 серогруппа) и 979/028 : H (28 серогруппа) вынести из генуса *Morganella morganii* и поместить их в генус *Reitterella reitteri* без пересчёта антигенной схемы *Morganellae*.

ANTIGENIC RELATIONSHIPS BETWEEN MORGANELLA MORGANII AND DIFFERENT GENERA OF ENTEROBACTERIACEAE

K. RAUSS, S. VÖRÖS

АНТИГЕННАЯ СВЯЗЬ МЕЖДУ MORGANELLA MORGANII И НЕКОТОРЫМИ ГЕНУСАМИ ENTEROBACTERIACEAE

К. РАУШШ, Ш. ВЕРЁШ

Авторы проводили систематические исследования по выявлению O-антигенной связи между *Morganella* и прочими генусами, относящимися в семью *Enterobacteriaceae*. На основе их данных можно установить наличие многочисленных связей. Большинство этих связей имеет a,b — a,c характер и низкого уровня, но были найдены и идентич-

ные O антигены. Идентичные O антигены встречались в следующем соотношении: *M. morganii* O10 — *P. hauseri* O12; *M. morganii* O23 — *P. hauseri* O17; *M. morganii* O8 — *R. rettgeri* O10; *M. morganii* O20: K1 — *E. coli* 0112ac: K66(B11); *M. morganii* O34 — *E. coli* O111 a,b: K58(B4). Была показана многократная антигенная связь в соотношении *M. morganii* O9 — *P. hauseri* O7 — *Citrobacter* O14 — *S. invernese* — *S. niarumbe* и *S. bergen*. Подчеркивается диагностическая важность антигенных связей и значение антигенохимических исследований этих реляций.

VERGLEICHENDE CHEMISCHE ANALYSE DER LIPOPOLYSACCHARID-ANTIGENE DER SHIGELLA SONNEI-MUTANTEN PHASE I UND II SOWIE R

T. KONTROR, O. WESTPHAL

СРАВНИТЕЛЬНЫЙ ХИМИЧЕСКИЙ АНАЛИЗ ЛИПОПОЛИСАХАРИДНЫХ АНТИГЕНОВ МУТАНТОВ I, II И R ФАЗЫ SHIGELLA SONNEI

Т. КОНТРОР, О. ВЕСТФАЛ

Из мутантов I(S), II и R фазы *Shigella sonnei* были извлечены серологически активные липополисахариды, которые затем подверглись сравнительному химическому исследованию. Авторы пришли к заключению, что между антигенной структурой липополисахаридов имеется очень большое сходство.

В основе каждого из трех полисахаридов лежит полигептозофосфатная цепочка, к которой присоединяются молекулы глюкозы (R) или глюкозы и галактозы (I и II фазы). Мутация S—R связана с обменом веществ галактозы. Причину резкого серологического расхождения между липополисахаридами I и II фаз нужно искать в более нежной структуре молекулы липополисахарида, тогда как серологический характер R мутантов объясняется отсутствием галактозы. Настоящие аналитические данные подтверждают то ранее предположение, что S — R мутация *Sh. sonnei* происходит на основе механизма, очень схожего с таковым у *Salmonella*.

Исследование так называемых L₁ фракций подтвердило, что последние состоят из нескольких компонентов, которые кроме нуклеиновых кислот содержат и полисахариды, построенные из маннозы и фруктозы.

CLINICAL AND SEROLOGICAL OBSERVATIONS ON AUTOGENOUS AND HETEROGENOUS VACCINATION IN STAPHYLOCOCCAL SKIN DISEASES

T. ANGYAL, A. LACZAY, K. CSAPÓ

КЛИНИЧЕСКИЕ И СЕРОЛОГИЧЕСКИЕ НАБЛЮДЕНИЯ, КАСАЮЩИЕСЯ АВТО- И ГЕТЕРОГЕННОЙ ВАКЦИНАЦИИ ПРИ СТАФИЛОКОККОВЫХ ЗАБОЛЕВАНИЯХ КОЖИ

Т. АНДЬЯЛ, А. ЛАЦАЙ, К. ЧАПО

49 больных, страдающих хронической и рецидивирующей стафилодермой, вакцинировались автовакциной, диффузной и компактной вакциной Smith-a, а также имеющейся в обращении поливалентной вакциной. В случае авто- и диффузной вакцины Smith-a был достигнут хороший терапевтический эффект, в отличие от компактной вакцины Smith-a и имеющейся в обращении поливалентной вакцины. Клинический эффект был подтвержден из иммунологических реакций фагоцитным индексом и опытами по пассивной защите мышей. Прямые и непрямые (Соомбс) гемагглютинины сывороток ни с гомологичными, ни с гетерологичными антигенами не совпадали с иммунитетом. Уровень некоторого изменения альфаантитоксина также остался ниже нормального уровня.

IN VITRO ANTIGEN FIXING CAPACITY OF ORGANS FROM IMMUNIZED RABBITS

M. KÁVAL, B. CSABA, L. KESZTYŰS

СПОСОБНОСТЬ ОРГАНОВ ИММУНИЗИРОВАННЫХ КРОЛИКОВ СВЯЗЫВАТЬ АНТИГЕН IN VITRO

М. КАВАИ, В. ЧАБА, Л. КЕСТЬЮШ

Для определения антител, связанных с тканевыми клетками, изучали отношение специфического связывания антигена и неспецифической адсорбции в зависимости от концентрации антигена. Было установлено, что неспецифическая адсорбция промыванием может быть понижена до минимума, тогда как иммун-специфическая связь не изменяется. На повышение дозы антигена связь тканевых антител к антигенам повышается и достигает максимума. Метод пригоден для приблизительно точного определения количества антител, присутствующих в тканях.

INTESTINAL MICROFLORA OF THE LARVAE OF ST. MARK'S FLY II. COMPUTER ANALYSIS OF INTESTINAL ACTINOMYCETES FROM THE LARVAE OF A BIBIO-POPULATION

I. SZABÓ, M. MARTON, L. FERENCZY, I. BUTI

ИНТЕСТИНАЛЬНАЯ МИКРОФЛОРА ЛАРВ БАРХАТНОЙ МАРТОВСКОЙ МУХИ

II. Компьютер-анализ кишечной лучисто-грибковой флоры одной популяции ларв

И. САБО, М. МАРТОН, Л. ФЕРЕНЦИ, И. БУТИ

Из экскрементов кишечника ларв *Bibio marci* L, относящихся к одной популяции, и «контрольной почвы» (Rendzina: A_H) всего было выделено 2557 штаммов лучистого грибка. По сравнительным исследованиям большое число видов лучистого грибка почвы в пищеварительном тракте подвергается значительной селекции. В кишечнике можно было наблюдать массовую встречаемость двух вариантов (с гладкой и с ворсистой поверхностью спор) лишь одного вида *Streptomyces* (*Streptomyces finlayi*), далее — присутствие нескольких других лучистых грибов, встречающихся спорадически и в небольшом количестве, способных переживать пассажи в кишечном тракте. Для установления представленной числовой пропорции во всеобщей флоре лучистого грибка отдельных видов *Streptomyces* была сделана попытка применить «вытесняющий» метод, основанный на физиологическом характере, отличающемся в случае отдельных видов.

INTESTINAL MICROFLORA OF THE LARVAE OF ST. MARK'S FLY III. COMPUTER ANALYSIS OF THE INTESTINAL ACTINOMYCETES-FLORA OF VARIOUS LARVAL POPULATIONS

I. SZABÓ, M. MARTON, L. FERENCZY, I. BUTI

ИНТЕСТИНАЛЬНАЯ МИКРОФЛОРА ЛАРВ БАРХАТНОЙ МАРТОВСКОЙ МУХИ

III. Сравнительный компьютер-анализ лучисто-грибковой флоры различных популяций ларв

И. САБО, М. МАРТОН, Л. ФЕРЕНЦИ, И. БУТИ

Авторы сравнили кишечную микрофлору 508 ларв, происходящих из 6 различных популяций *Bibio marci* L. Числовое соотношение стрептомицет в полной интестинальной микрофлоре обычно колебалось между 20—50%, но никогда не было менее 15%. Качественный состав кишечной *Streptomyces*-флоры у ларв, относящихся к идентичным

популяциям, оказался приблизительно одинаковым. Характерно было абсолютное преобладание какого-нибудь одного вида и наличие нескольких обычно редко встречающихся сопутствующих видов в различном количестве. На основе компьютер-анализа 84 свойств в кишечной микрофлоре особей, относящихся к различным популяциям ларв, виды *Streptomyces*, встречающиеся с большой частотой (доминирующие) — между прочим, всегда типичные обитатели почвы, — не были последовательно идентичными. Так, из изученных 319 штаммов в кишечнике 3 популяций ларв *Bibio* можно было наблюдать вариабельный *Streptomyces finlayi*, тогда как в лучисто-грибковой флоре кишечника дальнейших 3 популяций ларв наблюдались штаммы, относящиеся к другим видам, а именно: *Streptomyces olivaceus*, *Streptomyces antibioticus* или *Streptomyces aureofaciens*.

EFFECT OF MILK FROM COWS WITH MASTITIS ON THE ACTIVITY OF BUTTER CULTURE

S. SZAKÁLY

ДЕЙСТВИЕ МОЛОКА КОРОВ, БОЛЬНЫХ МАСТИТОМ, НА АКТИВНОСТЬ МАСЛЯНЫХ БАКТЕРИЙ

Ш. САКАЙ

Автор исследовал физические свойства культур масляных бактерий, продуцирующих молочную кислоту и аромат, в молоке, отобранном с помощью пробы *Whiteside* и обработанном температурой, происходящем от здоровых и больных коров. Было установлено, что конечная кислотная степень и общее количество бактерий культуры, приготовленной из молока коров, больных маститом, значительно ниже, чем в культуре, приготовленной из нормального молока. Культуры из молока больных коров практически без аромата, сгусток мягкий, рыхлый, горького вкуса, тошнотворный. В таком молоке подавление размножения видов *Str. lactis*, *Str. cremoris* и *Leukonostoc* происходит, повидимому, за счёт более низкой буферной возможности. Подавляющий эффект усиливается наличием термолабильных и термостабильных антител и частичным недостатком биофакторов.

Подавляющее действие, на основе определения кислотного содержания, прекращалось лишь тогда, когда в нормальном молоке содержалось менее 5% молока больных коров. С точки зрения образования аромата, вкуса и консистенции культуры различие проявлялось даже и тогда, когда ещё меньшее количество молока от коров, больных маститом, присутствовало в смеси.

ANTIGENIC STRUCTURE OF MYCOBACTERIUM KANSASII AND ITS VARIANTS

F. GIMPL, J. VÁNDOR

АНТИГЕННАЯ СТРУКТУРА MYCOBACTERIUM KANSASII И ЕГО ВАРИАНТОВ

Ф. ГИМПЛ, Й. ВАНДОР

Имунной диффузией, иммуноэлектрофорезом и иммунной абсорбцией было проведено исследование 12 штаммов *Mycobacterium kansasii*, среди них *scotochromogen*- и *albino*-вариантов. Антигенная структура штаммов оказалась единой независимо от их пигментообразования. Свои исследования авторы дополнили также бактериологическими и биохимическими методами. На основе опытов установлено, что виды могут быть хорошо определены биохимическими и иммунологическими методами. Распределение по *Ru-nyon-u*, основанное на пигментообразовании, фото- или скотохромогенность не достаточны для классификации атипичных микобактерий. Для классификации — кроме биохимических процессов — является основным сравнение антигенной структуры.

THE ANTIGENIC STRUCTURE OF RAT SARCOMA

G. LÁZÁR

АНТИГЕНЫ КРЫСИНОЙ САРКОМЫ

Г. ЛАЗАР

В сыворотке кроликов, гипериммунизированных экстрактом *Беневолентской* крысиной саркомы, методом радиальной двойной иммунной диффузии *Ouchterlony* можно показать многочисленные антитела, которые реагируют с различными белками крысиной плазмы. В кроликах, иммуноtolерантных к крысиной плазме, удалось получить такую противоопухолевую сыворотку, которая реагировала только с одним компонентом крысиной плазмы и с четырьмя компонентами опухолевого экстракта. Каждый из четырех компонентов обнаруживался в печени и селезенке, три — в почках и среди антигенов тонкого кишечника, два — в легких, один, распадаясь на два компонента, — в мышце сердца, и не было найдено ни одного компонента в поперечно-полосатой мускулатуре. Выявить такой антиген, который был бы характерен только для данной опухоли, не удалось.

THE EFFECT OF HEAT ON THE PHAGE SENSITIVITY OF STAPHYLOCOCCI

H. MILCH, V. G. LÁSZLÓ

ДЕЙСТВИЕ ТЕМПЕРАТУРНОЙ ОБРАБОТКИ НА ЧУВСТВИТЕЛЬНОСТЬ СТАФИЛОКОККОВЫХ ШТАММОВ К ФАГАМ

Х. МИЛХ, В. Г. ЛАСЛО

Среди 544 стафилококковых штаммов, не поддававшихся типизации основной и добавочной серией фагов, после температурной обработки 46,3% стали типизируемыми. В процессе изучения лизогенного состояния штаммов без обработки температурой 63,0%, после температурной индукции 75,1% штаммов оказались лизогенными. Исследуя лизисный спектр темперированных фагов, освободившихся спонтанно и под действием температуры, нашли, что *host range* высвободившихся на действие температурной индукции фагов на 50,3% был шире, чем таковой в случае спонтанно освободившихся темперированных фагов.

Определение фагового типа после обработки температурой, а также группирование, проводимое на основе лизисного спектра освободившихся темперированных фагов, оказались хорошо приемлемыми для эпидемиологических целей.

Авторам опытным путем удалось подтвердить их рабочую гипотезу, объясняющую изменяющуюся на действие температуры фаговую чувствительность. Двойным заражением необработанного штамма было доказано, что индуцированный под действием температуры темперированный фаг рекомбинировался с фагом, использованным для типизации. Этот рекомбинант, располагающий новыми «*host range*» свойствами, растворяет предварительно резистентную бактерию. Были подробно изучены лизогенные и литические свойства рекомбинированного фага.

CHANGES IN THE FATTY ACID COMPOSITION OF STAPHYLOCOCCUS AUREUS UNDER VARIOUS CULTURAL CONDITIONS

L. VÁCZI, I. RÉDAI, A. RÉTHY

ИЗМЕНЕНИЕ СОСТАВА ЖИРНЫХ КИСЛОТ STAPHYLOCOCCUS AUREUS ПРИ РАЗЛИЧНЫХ УСЛОВИЯХ ВЫРАЩИВАНИЯ

Л. ВАЦИ, И. РЕДАИ, А. РЕТИ

Состав жирных кислот *Staphylococcus aureus* зависит от окружающих условий. В случае понижения pH и усилении оксигенизации питательной среды повышается количество ненасыщенных жирных кислот. В культуре, содержащейся при 37 °C на питатель-

ной среде с pH 7,2, ненасыщенные жирные кислоты бактерий составляют 5,9% от общего количества жирных кислот; в то же время во встряхиваемой культуре — 10,4%, а в культуре с pH 6,0 — 9,4 или 12,2%.

При незначительном повышении или понижении температуры количество ненасыщенных жирных кислот существенно не изменяется.

На количество насыщенных и ненасыщенных жирных кислот бактерий в решающей степени влияет состав питательной среды. В питательной среде, содержащей 5% человеческой сыворотки, ненасыщенные жирные кислоты культивируемого штамма *Staphylococcus aureus* составляют 26,9% от общего количества жирных кислот; в противоположность этому на синтетической питательной среде бактерии продуцируют 5% ненасыщенных жирных кислот, а на питательной среде, обогащенной витаминами, — 10,3%.

In vivo, при выращивании штаммов *Staphylococcus aureus* в брюшной полости морской свинки, содержание их ненасыщенных жирных кислот составляет 1/3, или 36,2%, от общего количества жирных кислот.

При условиях размножения, близких к физиологическим, синтез жирных кислот в этих бактериях отклоняется в направлении ненасыщенных жирных кислот.

PROPERTIES OF ESCHERICHIA COLI SEROGROUP O18 STRAINS ISOLATED FROM AN OUTBREAK OF ENTERITIS AMONG NEWBORN INFANTS

M. HARASZTI, É. CZIRÓK

СВОЙСТВА ШТАММОВ ESCHERICHIA COLI, ОТНОСЯЩИХСЯ В СЕРОГРУППУ O18, ВЫДЕЛЕННЫХ ВО ВРЕМЯ ЭПИДЕМИИ ЭНТЕРИТА СРЕДИ НОВОРОЖДЕННЫХ

М. ХАРАСТИ, Е. ЦИРОК

Авторы знакомят с изучением штамма *E. coli*, выделенного во время эпидемии энтерита среди новорожденных. На основе серологических исследований штамм может быть охарактеризован следующей антигенной формулой: O18a, 18c: K77(B21):H7. Энтерит в виде эпидемии, вызванный этим штаммом, в Венгрии до этого не наблюдался.

INCIDENCE OF CRYPTOCOCCUS SPECIES IN URBAN AIR

Gy. VÖRÖS-FELKAI

ВСТРЕЧАЕМОСТЬ В ГОРОДСКОМ ВОЗДУХЕ ВИДОВ CRYPTOCOCCUS

ДЬ. ВЁРЁШ-ФЕЛКАИ

Автор, исследуя в течение 18 месяцев флору дрожжевого гриба городского воздуха, выделил 329 штаммов белого дрожжевого гриба. 47,1% штаммов оказалась способной образовывать крахмал.

Было проведено точное идентифицирование 100 крахмалообразующих штаммов. Все 100 относились в генус *Cryptococcus albidus* (SAITO) SKINNER 74, *Cryptococcus diffluens* (ZACH) LODDER и KREGER VAN RY 17, *Cryptococcus laurentii* (KUFFERATH) SKINNER 9. *Cryptococcus neoformans* (SANFELICE) VUILLEMIN не встретился ни в одном из случаев. Встречаемость видов по месяцам была равномерной, расхождения по временам года не наблюдалось.

Так как эти виды из воздуха могут попасть на поверхность человеческого организма и внутрь его, в случае их наличия в исследуемом материале больных, подозрительных на микоз, необходимо серьезно обсудить, играют ли они какую-нибудь этиологическую роль в заболевании.

SUSCEPTIBILITY OF THE DOMESTIC SWINE TO INFLUENZA B VIRUS

Gy. TAKÁTSY, J. ROMVÁRY, E. FARKAS

ВОСПРИИМЧИВОСТЬ ДОМАШНИХ СВИНЕЙ К ВИРУСУ ГРИППА В

ДЬ. ТАКАЧИ, Й. РОМВАРИ, Е. ФАРКАШ

Авторы провели эксперимент по интраназальному заражению двух поросят свежим штаммом вируса гриппа В. У одного поросёнка сразу же после заражения поднялась температура, а впоследствии в крови можно было выявить нейтрализующие и подавляющие гемагглютинацию антитела против гомологичного штамма. Повышение температуры наблюдалось и у одной контактной свиньи, но в этом случае были обнаружены только подавляющие гемагглютинацию антитела, нейтрализующие — нет.

Были исследованы пробы крови от 924 свиней 8 хозяйств, среди которых наблюдались гриппоподобные заболевания, возникшие во время или после эпидемии человеческого гриппа. В 4 хозяйствах у 8—24% свиней выявили подавляющие гемагглютинацию антитела против свежевыделенного штамма гриппа В.

EFFECT OF THE PHYSICO-CHEMICAL PARAMETERS OF STREPTOCOCCUS PYOGENES CULTURES ON THE FORMATION OF O-STREPTOLYSIN

I. THE ROLE OF GLUCOSE CONCENTRATION AND pH IN THE FORMATION OF O-STREPTOLYSIN

J. BÖSZÖRMÉNYI, A. VERESS, I. FÜVESSY

ВЛИЯНИЕ ФИЗИКО-ХИМИЧЕСКИХ ПАРАМЕТРОВ КУЛЬТУРЫ STREPTOCOCCUS PYOGENES НА ВОЗНИКНОВЕНИЕ О-СТРЕПТОЛИЗИНА

I. Роль концентрации глюкозы и pH в образовании О-стрептолизина

Й. БЁСЁРМЕНЬИ, А. ВЕРЕШШ, И. ФЮВЕШШИ

Из питательных сред, содержащих 0,12—3,0% глюкозы, на 1%-ной питательной среде авторы достигли максимального числа бактерий. Отклонение от этой концентрации в сторону повышения или понижения одинаково неблагоприятно сказывалось на количестве бактерий. Скорость размножения в экспоненциальном периоде ограничивалась концентрацией глюкозы ниже 0,5%; содержание глюкозы выше этого уровня влияния не оказывало. С точки зрения образования О-стрептолизина оптимальными были найдены концентрация глюкозы, поддерживаемая в течение всего периода культивирования около 1%, и нейтральное значение pH. Концентрация глюкозы выше или ниже 1% в большей степени понижала образование О-стрептолизина, чем общее число бактерий.

EFFECT OF THE PHYSICO-CHEMICAL PARAMETERS OF STREPTOCOCCUS PYOGENES CULTURES ON THE FORMATION OF O-STREPTOLYSIN

II. THE ROLE OF REDUCING AGENTS IN THE FORMATION OF O-STREPTOLYSIN

I. FÜVESSY, J. BÖSZÖRMÉNYI, A. VERESS

ВЛИЯНИЕ ФИЗИКО-ХИМИЧЕСКИХ ПАРАМЕТРОВ КУЛЬТУРЫ STREPTOCOCCUS PYOGENES НА ВОЗНИКНОВЕНИЕ О-СТРЕПТОЛИЗИНА

II. Роль редуцирующих агентов в образовании О-стрептолизина

И. ФЮВЕШШИ, Й. БЁСЁРМЕНЬИ, А. ВЕРЕШШ

При различных экспериментальных условиях было исследовано действие натрий-тиогликолата, NaHCO_3 , дитионита натрия, глутатиона, метионина, тиамина, цистина, цистеина и аскорбиновой кислоты на рост штамма H 46A, относящегося в С группу

Streptococcus pyogenes и на образование О-стрептолизина. Перечисленные вещества не влияли на скорость удельного роста штамма Н 46А. Образование О-стрептолизина в небольшой степени повышалось цистеином, а также комбинацией цистин + аскорбиновая кислота и значительно усиливалось под влиянием комбинации цистеин + аскорбиновая кислота.

При использовании для продукции О-стрептолизина международных стандартных штаммов С203, S84, Richards, относящихся в группу А, а также штамма Тр 21 из группы С и комбинация цистеин + аскорбиновая кислота только в небольшой степени повышала образование О-стрептолизина, но на скорость их удельного роста влияния не оказывала.

EFFECT OF ENDOTOXIN ON THE SERUM LEVEL OF COMPLEMENT COMPONENTS

G. FÜST, P. KERTAI

ВЛИЯНИЕ ЭНДОТОКСИНА НА УРОВЕНЬ КОМПОНЕНТОВ КОМПЛЕМЕНТА СЫВОРОТКИ

ДЬ. ФЮШТ, П. КЕРТАИ

Было изучено влияние эндотоксина *S. typhosa* на уровень комплемента и его компонентов в сыворотке кроликов. Авторы установили, что спустя 48 часов после дачи эндотоксина общий уровень комплемента, а также титр С'2 и С'4 возросли в два раза, тогда как уровень двух других компонентов не изменился. В животных, толерантных к эндотоксину, повышения уровня комплемента не произошло.

THE ANTIGEN-FIXING CAPACITY ON TISSUES OF SENSITIZED GUINEA PIGS

M. KÁVAI, B. CSABA, S. JUSUPOVA, L. KESZTYŰS

АНТИГЕНСВЯЗЫВАЮЩАЯ СПОСОБНОСТЬ ТКАНЕЙ СЕНСИБИЛИЗИРОВАННЫХ МОРСКИХ СВИНОК

М. КАВАИ, Б. ЧАБА, Ш. ЮСУПОВА, Л. КЕСТЬЮШ

Легкие и сердце активно сенсibilизированных морских свинок, убитых во время анафилактического шока, по граммам иммуноспецифически связывали 0,5 — 1,5 $\mu\text{gN}^{51}\text{Cr}$ -овальбуминного антигена. Метод *in vitro*, разработанный авторами, применим для количественного определения антигенсвязывающей способности тканей активно сенсibilизированных морских свинок. В случае пассивной сенсibilизации метод не был пригоден. На основе результатов авторы в патогенезе шока у морских свинок наряду с легкими приписывают роль и сердцу.

THE MECHANISM OF ANTIBACTERIAL ACTION OF RAPHANIN

J. MOLNÁR

МЕХАНИЗМ АНТИБАКТЕРИАЛЬНОГО ДЕЙСТВИЯ РАФАНИНА

Й. МОЛНАР

Рафанин, антибактериальное вещество, содержащееся в семенах редьки, подавляет некомпетитивным способом активность SH-энзимов, а именно уреазы и сукцинодегидрогеназы. С другой стороны, это соединение не влияет на активность энзимов, не содержащих сульфгидрильной группы, как например, щелочная фосфатаза и трансаминаза глютаминовой и оксалукусусной кислот. На основе полученных результатов антибактериальное действие рафанина может быть объяснено подавлением сульфгидрильных энзимов бактерий.

THE EFFECT OF HAEMOCOAGULASE (REPTILASE) ON THE LOCAL SHWARTZMAN PHENOMENON

S. TÓTH, L. KASSAY, E. BARZÓ, T. SZILÁGYI

ДЕЙСТВИЕ РЕПТИЛАЗЫ НА ЛОКАЛЬНОЕ ЯВЛЕНИЕ SHWARTZMAN-A

Ш. ТОТ, Л. КАШШАИ, Е. БАРЗО, Т. СИЛАДЫИ

Авторы вызывали на кроликах локальное явление Shwartzman-a. 1 мг рептилазы, дозированный до или спустя два часа после дачи вызывающей (challenge) дозы эндотоксина, выразительно усиливал геморрагию и некроз. В опытах *in vitro* рептилаза не влияет на активность комплемента.

IDENTIFICATION OF SHIGELLAE ON THE BASIS OF SELECTIVE SENSITIVITY

B. SERÉNYI

ИДЕНТИФИКАЦИЯ ШИГЕЛЛ НА ОСНОВЕ ИХ СЕЛЕКТИВНОЙ ЧУВСТВИТЕЛЬНОСТИ

Б. ШЕРЕНЬ

В чувствительности членов семьи *Enterobacteriaceae* можно выявить характерные расхождения, если использовать диски из фильтровальной бумаги, пропитанные соответствующими химическими соединениями: малахитовый зелёный, кристаллический фиолетовый, сафранин, фусцин основной, Janus green, кобальтовый нитрат, сульфат цинка. Этим методом просто и надежно могут быть отождествлены прежде всего культуры *Shigella*, чувствительные к сафранину и основному фуксину.

CHANGE IN THE PHAGE TYPE OF STAPH. LOCOCCUS AUREUS AS A RESULT OF LYSOGENIZATION

J. LANTOS

ИЗМЕНЕНИЕ ФАГОВОГО ТИПА ШТАММОВ STAPHYLOCOCCUS AUREUS НА ДЕЙСТВИЕ ЛИЗОГЕНИЗАЦИИ

Й. ЛАНТОШ

По случаю контрольного обследования в условиях больницы внутри одного и того же коллектива наряду с часто встречающимися штаммами 29/52/52A/80/52B и 52B было выявлено много нетипизирующихся штаммов *Staphylococcus*. Для отождествления нетипизирующихся штаммов использовали опытные фаги; и на основе типизации этими фагами и «профагтипизации» большинство штаммов показало тождественную фаговую картину.

Фагами, выделенными из нетипизирующихся штаммов, лизогенизировали штаммы типа 29/52/52A/80/52B и 52B; в результате были получены штаммы, резистентные к основным фагам.

С помощью опытных фагов штаммы были отнесены в последующие группы, и благодаря искусственной лизогенизации можно было следить за возможностями формирования нетипизирующихся штаммов.

**COMPARATIVE STUDY ON THE INTERFERON PRODUCTION BY
THE LEUKOCYTES OF HEALTHY AND LEUKAEMIC SUBJECTS**

GY. HADHÁZY, L. GERGELY, F. D. TÓTH, GY. SZEGEDI

**СРАВНЕНИЕ СПОСОБНОСТИ ЛЕЙКОЦИТОВ ЗДОРОВЫХ И СТРАДАЮЩИХ
ЛЕЙКЕМИЕЙ ЛИЦ ПРОДУЦИРОВАТЬ ИНТЕРФЕРОН**

ДЬ. ХАДХАЗИ, Л. ГЕРГЕЙ, Ф. Д. ТОТ, ДЬ. СЕГЕДИ

Способность лейкоцитов, происходящих от больных с миелоидной лейкемией, продуцировать интерферон выше, чем таковая в случае здоровых доноров. Лейкоциты больных лейкемией на действие вируса *Newcastle* продуцируют больше интерферона, чем на действие вируса *Sendai*. С другой стороны, здоровые лейкоциты на действие обоих вирусов продуцируют приблизительно одинаковое количество интерферона.

Продукцию интерферона лейкоцитами больных с миелоидной лейкемией актино-мицин-Д подавляет в меньшей степени, чем это имеет место в случае здоровых лейкоцитов.

Лейкоциты больных с хронической лимфоидной лейкемией продуцируют одинаковое или меньшее по сравнению с лейкоцитами здоровых лиц количество интерферона.

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